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Integrative multi-method survey for terrestrial vertebrate inventories

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ABSTRACT

Biodiversity monitoring is critical for detecting environmental change, protecting threatened species, and guiding evidence-based conservation decisions. However, traditional approaches, such as direct observations, are often constrained by high labor demands, extended survey durations, and limited spatial coverage. To address these limitations, a large-scale, non-invasive assessment of terrestrial vertebrates was implemented in the Gaoligongshan National Nature Reserve, a biodiversity hotspot in southwestern China. This assessment integrated three independent detection techniques, including camera trapping, leech invertebrate-derived DNA (iDNA), and aquatic environmental DNA (eDNA) profiling, to maximize species detection and reduce method-specific biases. Collectively, the combined framework identified 102 wild terrestrial vertebrate species, including 60 mammals, 27 birds, 14 amphibians, and one reptile. Water eDNA achieved the broadest coverage, identifying 71 species (70% of the total), whereas both camera traps and leech iDNA each recorded 32 species. Only eight taxa were detected across all three platforms, reflecting their strong complementarity in capturing species assemblages. This multi-modal framework illustrates the effectiveness of integrating molecular and imaging-based approaches to produce high-resolution biodiversity inventories, thereby reinforcing the scientific basis for evaluating conservation outcomes and guiding the development of adaptive management strategies in complex ecosystems.

Keywords: Baoshan area of Gaoligong Mountains; Camera traps; Leech iDNA; Water eDNA; Terrestrial vertebrates

INTRODUCTION

Biodiversity monitoring is essential for maintaining ecosystem integrity, evaluating environmental health, and guiding the development of robust management strategies (Lindenmayer & Likens, 2010; Sutherland et al., 2013). It establishes crucial baseline data on species distributions, population dynamics, and ecosystem functions, serving as the foundation for evidence-based conservation planning (Chapin III et al., 2000). However, traditional approaches, typically reliant on expert-led field surveys, are constrained by high labor requirements, observer bias, and limited spatial and temporal coverage (Beever, 2006). These limitations are particularly acute in remote or ecologically inaccessible areas, where sustaining large-scale, long-term monitoring is logistically challenging (Lindenmayer et al., 2022).

Advances in technology have transformed biodiversity assessment, enabling broader coverage, higher resolution, and greater efficiency. Infrared cameras, satellite remote sensing, and DNA-based techniques (Stephenson, 2020) offer new opportunities for biodiversity to be surveyed with unprecedented scope and precision. Camera traps have become a cornerstone of terrestrial vertebrate monitoring over the past two decades (Burton et al., 2015), while environmental DNA (eDNA) analysis has rapidly emerged as a powerful tool, particularly in aquatic ecosystems (Afonso et al., 2024; Harper et al., 2019; Huang et al., 2022; Sahu et al., 2023; Schenekar, 2023). In terrestrial ecosystems, however, the most effective environmental medium for biodiversity monitoring remains unresolved, with studies indicating that soil, water, air, and invertebrate-derived DNA (iDNA) each

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hold considerable potential (Mena et al., 2021; Sahu et al., 2023; Van Leeuwen & Michaux, 2023; Weiskopf et al., 2023).

No single monitoring method can fully capture biodiversity patterns due to inherent detection biases, taxonomic limitations, and environmental constraints. Consequently, integrating complementary approaches can significantly enhance detection breadth, improve accuracy, and produce more representative biodiversity inventories (Harper et al., 2019). However, effective integration requires rigorous comparative evaluation of the capabilities and limitations of individual methods to optimize multi-technology monitoring frameworks.

Although various studies have compared the effectiveness of different monitoring methods, such as camera trapping vs. water eDNA, camera trapping vs. soil eDNA, camera trapping vs. iDNA, and eDNA vs. iDNA (Carvalho et al., 2022; Gogarten et al., 2020; Leempoel et al., 2020; Lyet et al., 2021; Rodríguez-Castro et al., 2023; Tilker et al., 2020), most have been limited to pairwise analyses. Comprehensive evaluations incorporating three or more independent techniques remain rare, restricting the ability to fully realize the benefits of integrated monitoring strategies.

The southwestern mountains of China, recognized as a global biodiversity hotspot (Mittermeier et al., 2005), have long been a focus of intensive scientific research and conventional species surveys. Nevertheless, biodiversity monitoring in this region remains heavily dependent on camera trapping, with minimal integration of complementary methods, limiting the scope and completeness of ecological assessments. Moreover, serving as a critical ecological barrier and a priority zone for ecological restoration in western China, the southwestern mountains are increasingly subject to the dual challenges of large-scale development and climate change. These pressures threaten the rich biodiversity of the area and the capacity of ecosystems to sustain essential services. Establishing a multi-technology integrated monitoring system capable of generating comprehensive biodiversity data is therefore essential to support effective conservation and management efforts in this ecologically important landscape.

This study examined terrestrial vertebrate diversity in the Gaoligong Mountains, one of the most biodiverse regions in southwestern China (Zhou et al., 2023), through an integrated monitoring framework combining camera trapping, leech-derived iDNA, and water eDNA. This approach was designed to address the limitations inherent in single-method monitoring surveys, enhancing both the efficiency and accuracy of biodiversity assessments. The findings of this study aim to provide a practical foundation for establishing a multi-technology, integrated biodiversity monitoring system to support long-term conservation and management across the southwestern mountains of China.

MATERIALS AND METHODS

Study area

The study was conducted in the Baoshan region, covering central and southern sections of the Gaoligongshan National Nature Reserve in Yunnan Province, China (N24°56′–N25°20′, E98°34 ′ –E98°50 ′). The area lies within the subtropical monsoon climate zone of low-latitude mountains, with a mean annual temperature and precipitation of approximately 16°C and 1 375 mm, respectively. Both temperature and precipitation decline progressively with increasing elevation. The altitudinal range extends from 210 m to 5 128 m above sea level, encompassing a diverse array of ecosystems from lowland tropical forests to high-altitude alpine meadows. Vegetation exhibits a distinct vertical stratification, with evergreen broadleaf forests dominating at lower elevations, gradually transitioning into bamboo forests, coniferous forests, and shrub meadows as altitude increases. The region supports a rich and distinctive wildlife assemblage, with high faunal diversity. Lowland and river valley areas are also inhabited by various ethnic minority communities, where human activity and disturbance are more significant.

Camera trap survey

Camera traps were initially deployed in the Baoshan region of the Gaoligongshan National Nature Reserve in 2017. For the present study, analyses focused on data collected between 2022 and 2023 from two monitoring sites: Baihualing (BS) in the north and Laobaoteng (LBT) in the south. Each site was equipped with 30 camera units (model EREAGLE-E1B, Dongfang Hongying) positioned along an elevation gradient, resulting in a total of 60 monitoring locations (Figure 1). These sites encompassed habitats ranging from low-elevation dry river valleys to high-elevation alpine meadows, with a minimum distance of 800 m between adjacent cameras. Camera trap elevations ranged from 1 535 m to 3 272 m, with a mean elevation of 2 432 m.

Cameras were installed at sites showing clear evidence of terrestrial vertebrate activity, such as tracks, scats, and feeding signs, as well as along likely movement corridors. Each unit was mounted approximately 1 m above ground level, positioned away from prominent human activity paths to minimize disturbance, and set to automatic trigger mode. Sensitivity was set to “low” to reduce false triggers caused by environmental factors such as wind-blown vegetation or precipitation.

Upon activation, each trigger event recorded three still images and one 15 s video, with a 3 s interval between images. Equipment was checked regularly to retrieve data and replace batteries and memory cards as needed. Image and video data were manually screened by trained mammalogists. Identifications to the species level were made only when certainty was high; ambiguous records (e.g., certain small squirrels or birds) were excluded. False captures caused by non-animal movement were also excluded. For repeated captures of the same species at the same location, only records separated by more than 1 h were considered independent events to avoid overestimation of species occurrences.

Species identification was based on morphological characteristics, including size, pelage coloration, and behavior, and verified against existing species catalogs and field guides. Conservation status was assigned using the most recent version of the International Union for Conservation of Nature (IUCN) Red List (IUCN, 2021; https://www.iucnredlist.org), with categories including Critically Endangered (CR), Endangered (EN), Vulnerable (VU), and Near Threatened (NT). All camera trap records were organized into species-specific datasets.

To assess inter-annual consistency in monitoring, the coefficient of determination (R^2) was calculated for species-specific detection probabilities (per camera-day) at identical sites between 2022 and 2023 (Supplementary Figure S1). The resulting high R^2 value (0.972) indicated strong correlation in relative detectability rankings across species, independent of

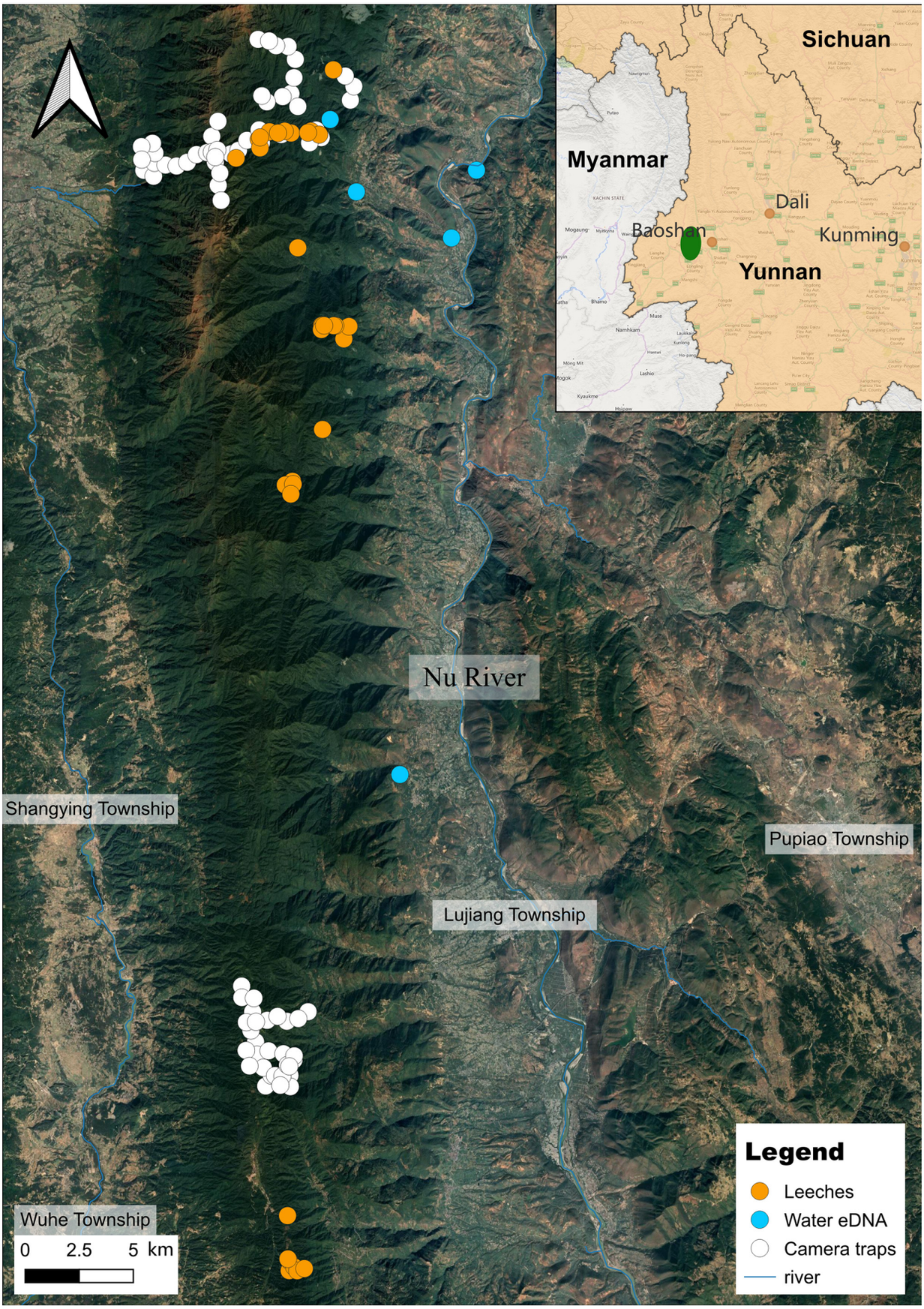

Figure 1 Distribution of sampling points for monitoring terrestrial vertebrates in the Baoshan section of the Gaoligongshan National Nature Reserve, Yunnan, China

Orange dots represent leech eDNA sampling points; blue dots represent water eDNA sampling points; white dots represent infrared camera locations.

potential variation in community composition. Based on this high consistency, only the 2023 dataset was used for subsequent analyses.

Environmental DNA survey

Sample collection:

1) Leech-derived iDNA samples

Leeches were collected between 23 August and 24 September 2022, and between 15 June and 1 September 2023. Sampling was conducted by rangers from the Baihualing, Saige, and Nankang forest protection stations during routine patrols. Each specimen was preserved in a collection tube containing RNALater, accompanied by detailed metadata, including geographic coordinates (latitude, longitude, and elevation), collection date, affiliated protection station, and collector name. A total of 847 leeches were obtained, including 223 in 2022 and 624 in 2023. For molecular analysis, these were pooled into 35 composite samples according to collection date and site (Figure 1).

2) Water eDNA samples

Water samples were collected between 23 and 29 May 2022 along three rivers in the Baoshan region, including the Manggang and Tangxi rivers located in the northern Baihualing monitoring site and Mangzhao River located centrally. To capture upstream species diversity, sampling points were positioned in both the middle-upper and lower reaches of the Manggang and Tangxi rivers, resulting in four sampling sites, while a single site was established in the middle reaches of the Mangzhao River (Figure 1).

At each site, 30 L of water was collected, with approximately 6 L filtered per membrane. This produced five replicates per site, except at the Mangzhao River, where high turbidity limited filtration to 5 L per filter, yielding six replicates. In total, 26 water samples were collected. Field filtration was performed using closed butterfly filters (custom-made by Kobot Company; PES membrane; pore size: 0.8 μm) and an electric peristaltic pump (Jieheng, model: JIHPUMP, BT-600EA/253Yx), with the volume filtered recorded for each sample. To prevent contamination, all equipment was cleaned with 5% sodium hypochlorite solution, during which disposable gloves were worn, and a negative control sample included at each site. Filters were preserved by adding 2 mL of Longmire solution (Spens et al., 2017).

Sampling site selection and scheduling for the three monitoring approaches were based on ecological conditions and methodological requirements. Camera traps were deployed year-round along high-elevation animal movement corridors to maximize detection probability. Leech iDNA sampling was conducted along ranger patrol routes during the rainy season, relying on leech feeding behavior to capture host DNA. Water eDNA samples were collected before the onset of the rainy season to reduce dilution effects. Although the exact sampling sites for the three methods did not completely overlap, spatial coverage was carefully planned to encompass the full range of habitats in the study area, thereby increasing the probability of detecting the widest possible range of species.

Molecular experiments:

DNA extraction: Leech samples were processed and digested following Ji et al. (2022), while water samples were processed and digested following Spens et al. (2017). After overnight digestion, DNA from both leech and water sample digests was purified using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocols, including DNA adsorption, washing, and elution. The purified DNA was divided into two portions: one stored at −20°C for long-term storage and the other at 4°C for immediate use.

Polymerase chain reaction (PCR) amplification and sequencing: Metabarcoding was performed using two mitochondrial rRNA primer pairs: 12S rRNA vertebrate universal primers 12SV05 (5′-ACTGGGATTAGATACCCC-3′ and 5′-YRGAACAGGCTCCTCTAG-3′) (Riaz et al., 2011) and 16S rRNA mammal universal primers 16Smam (5′-CGGTTGGGGTGACCTCGGA-3′ and 5′-GCTGTTATCCCTAGGGTAACT-3′) (Taylor, 1996). All primers were synthesized with an 8-base tag sequence at the 5′ ends. The PCR volume (20 μL) contained 1 μL of DNA template, 0.2 μM primers, 0.2 mM dNTPs, 0.6 U Ex Taq HotStart DNA polymerase (TAKARA Biosystems, Dalian, China), and 1× PCR buffer. Cycling conditions were: initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 45 s, followed by a final extension at 72°C for 7 min. Each sample was amplified in triplicate for each gene using different dual-tag pairs. PCR products were verified by 2.5% agarose gel electrophoresis, with target sizes of 81–117 bp for 12S and 82–150 bp for 16S (excluding primers). Successfully amplified products were quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, New York, USA), then pooled into six 12S libraries and six 16S libraries. Libraries were sent to Novogene (Beijing, China) for PCR-free library construction and sequencing on the Illumina NovaSeq 6000 platform (PE150).

Contamination control: All molecular experiments were conducted in an eDNA-dedicated laboratory, maintained at ISO 5 clean air standards with positive pressure. The facility was divided into pre-processing, DNA extraction, reagent preparation, and PCR areas, with strict adherence to unidirectional workflows to minimize contamination risk. Work surfaces were cleaned with 5% sodium hypochlorite and irradiated with UV light overnight. Negative controls were included in both DNA extraction and PCR amplification, processed identically to samples, and subjected to high-throughput sequencing to detect potential contamination sources and inform filtering thresholds during bioinformatic analysis.

Bioinformatics analysis:

The quality of raw sequencing data from each library was first evaluated using MultiQC (Ewels et al., 2016) to confirm that the average base quality score exceeded 20. Subsequent preprocessing and quality control were performed in FASTP (Chen et al., 2018), which included removal of residual adapter sequences and low-quality bases, as well as the merging of paired-end reads. An HTML report was generated for each library to provide a detailed overview of data quality before and after processing.

Merged reads were assigned to their respective tag pairs using the 'sort.py' function in the DAMe pipeline (Zepeda-Mendoza et al., 2016), based on the 5′ and 3′ tag sequences of merged reads. After tag assignment, the average number of sequences per PCR replicate was as follows: leech iDNA, 389 275 sequences for 12S and 329 890 for 16S; water eDNA, 472 135 sequences for 12S and 439 875 for 16S.

Filtering was subsequently performed with the 'filter.py' function in DAMe, with parameters determined from negative control data to remove tag-jumping artifacts, erroneous

sequences, and potential contamination. For both 12S and 16S datasets, the *y* parameter was set to 2, retaining only sequences present in at least two of the three PCR replicates. For leech-derived 12S and 16S data, the *t* parameter was set to 2, reflecting lower complexity and higher stability; for water-derived 12S and 16S data, the *t* parameter was set to 13 and 9, respectively. Here, *t* indicates the minimum number of sequence copies required in each replicate. After filtering, all DNA extraction and PCR negative controls were cleared of sequences, except for trace human DNA reads in some water eDNA controls. As the study focused exclusively on non-human vertebrates, human DNA read contamination was ignored.

Post-filtering, VSEARCH v.2.9.0 (Rognes et al., 2016) was used to remove chimeric sequences. Remaining reads were preliminarily clustered into operational taxonomic units (pre-OTUs) using sumaclust (Mercier et al., 2013) at a 99% similarity threshold. Pre-OTUs with high sequence similarity and consistent sample distribution were merged into final OTUs using the R package LULU v.0.1.0 (Frøslev et al., 2017). Taxonomic assignment of OTU representative sequences was performed in PROTAX (Somervuo et al., 2016, 2017) using a reference database based on MIDORI (Machida et al., 2017), supplemented with updated mitochondrial gene sequences of Gaoligong vertebrates from NCBI GenBank and unpublished mitochondrial genomes generated at the Kunming Institute of Zoology (to avoid copyright issues, these sequences are unpublished). A vetted species list for Gaoligong vertebrates was compiled by taxonomic experts from the Kunming Institute of Zoology (Li et al., 2024; Wang et al., 2024; Wu et al., 2024).

Data from leech iDNA and water eDNA analyses are summarized in Table 1. For species-level comparisons, OTUs without species-level identification were removed. OTUs identified as human, domestic animals, or fish were also removed. Because the targeted DNA fragments could not differentiate between domestic animals and their wild counterparts (e.g., domestic pigs and wild boars, dogs and wolves, and chickens and wildfowl), these OTUs were classified as domestic species in the DNA dataset. Finally, OTUs identified as the same species were merged to generate the final species-level dataset.

Comparative analysis of data:

All statistical analyses were conducted in R v.4.4.2, using multiple packages for various analytical tasks. Species detection probabilities were calculated as the frequency of occurrence for each species across monitoring sites, with row-wise operations implemented using the 'apply()' function. Correlation between 2022 and 2023 camera trap data was evaluated via linear regression analysis using the 'lm()' function, with the correlation coefficient and R^2 values used to assess goodness of fit. Species accumulation curves were generated with the 'iNEXT()' function from the iNEXT package (Chao et al., 2014), using 'incidence_raw' data and q=0 to evaluate species diversity coverage for each monitoring method. Venn and Euler diagrams were generated using the ggVennDiagram (Gao et al., 2024) and eulerr (Micallef & Rodgers, 2014) packages, respectively. The former illustrates species overlap between genetic markers, while the latter depicts shared and unique species distributions across different monitoring methods. Species composition analysis was performed using ggplot2 to generate stacked bar plots, highlighting differences in species distribution and sources across detection methods.

RESULTS

Sampling intensity and sufficiency

In 2023, the camera trap monitoring data included 53 active sites, yielding a total of 16 041 camera-days suitable for comparison with DNA-based data. Across the monitoring period, cameras recorded 17 407 valid videos, capturing mammals, as well as humans, livestock, and birds. In the final DNA dataset, leech-derived samples comprised 25 valid samples from 25 sampling points, and water-derived samples comprised 26 valid samples from five sampling points. For leech iDNA data, the proportion of valid wild terrestrial vertebrate sequences was 53.8% for 12S (17 418 185/32 368 293) and 74.1% for 16S (14 097 685/19 032 315). For water eDNA, the valid proportion was 15.2% for 12S (4 740 110/31 220 460) and 46.0% for 16S (13 263 051/28 857 156).

Species rarefaction curve analysis (Figure 2) indicated that detected species richness increased with the duration of camera deployment, the number of monitoring sites, and the number of DNA samples. Species richness from camera traps reached a plateau when monitoring spanned 20 months and included 25 sites, indicating that sampling saturation had been achieved. In contrast, richness from leech iDNA and water eDNA continued to increase, suggesting that these DNA-based approaches had not yet reached saturation and that additional sampling would likely detect more species.

General overview of biodiversity

Across all three monitoring approaches—camera traps, leech

Table 1 Overview of DNA data and total number of reads

	Leech iDNA 12S	Leech iDNA 16S	Water eDNA 12S	Water eDNA 16S
Total number of reads	32 368 293	19 032 315	31 220 460	28 857 156
Number of OTUs	239	116	149	134
Number of OTUs matched to species level	222	98	100	131
Number of species	34	26	80	69
Proportion of human reads	3.25%	4.56%	7.33%	23.56%
Proportion of fish reads	0.33%	0.00%	55.29%	3.64%
Proportion of domestic animal reads	10.53%	18.57%	14.21%	18.13%
Proportion of amphibian and reptile reads	22.85%	16.74%	10.80%	9.09%
Proportion of mammal* reads	24.73%	60.00%	11.04%	45.55%
Proportion of bird* reads	12.86%	0.02%	1.33%	0%
Proportion of NA reads	25.45%	0.11%	0%	0.03%

Note: *Reads for mammals and birds exclude domestic species such as cattle, sheep, pigs, chickens, etc.

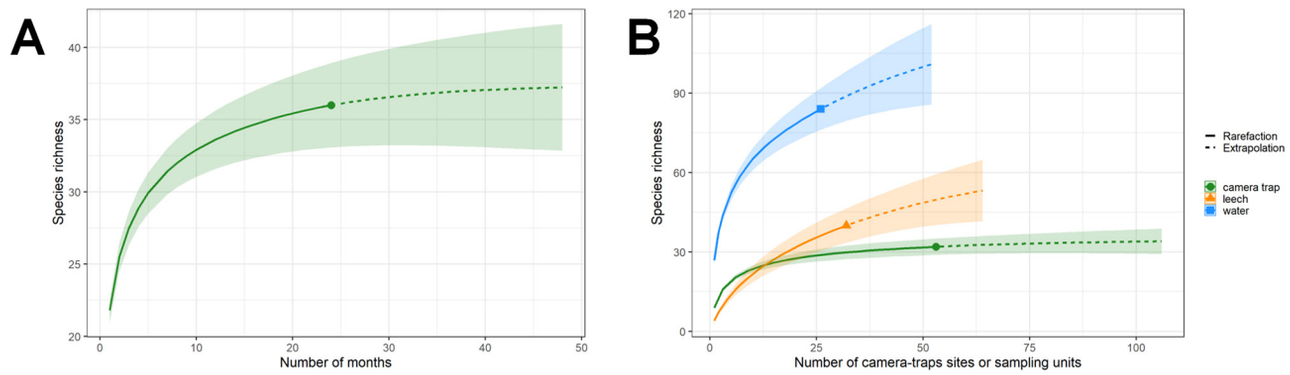

Figure 2 Rarefaction curves for different methods

Green represents camera trapping, blue represents water eDNA, and orange represents leech iDNA. Solid lines for each method indicate actual observed species richness, dashed lines represent predicted species richness through extrapolation, and colored shaded areas indicate 95% confidence intervals for predicted species richness. A: Rarefaction curve for species richness over time for camera traps. X-axis represents number of months; y-axis represents species richness. B: Rarefaction curves for three detection methods. X-axis represents number of camera-trap sites or sampling units; y-axis represents species richness.

iDNA, and water eDNA —a total of 102 wild terrestrial vertebrate species were recorded in the Baoshan region of the Gaoligongshan National Nature Reserve (Supplementary Figure S2), including 60 mammal species (belonging to 7 orders, 19 families, and 39 genera), 27 bird species (from 7 orders, 15 families, and 24 genera), 14 amphibian species (from 2 orders, 6 families, and 9 genera), and one reptile species (from one order, one family, and one genus). According to the IUCN Red List, one species was listed as CR, 2 as EN, 7 as VU, and 6 as NT.

Only 8 species were detected by all three methods: leopard cat (*Prionailurus bengalensis*), red muntjac (*Muntiacus vaginalis*), stump-tailed macaque (*Macaca arctoides*), Assamese macaque (*Macaca assamensis*), brush-tailed porcupine (*Atherurus macrourus*), Malayan porcupine (*Hystrix brachyura*), Yunnan giant flying squirrel (*Petaurista yunanensis*), and northern tree shrew (*Tupaia belangeri*). 16 species were detected by both camera traps and leech iDNA, 13 species were detected by both camera traps and water eDNA, and 12 species were detected by both leech iDNA and water eDNA (Figure 3; Supplementary Figure S2).

Detection results of the three methods

Across all sampling points, the median number of species detected per sample was 8 for camera traps (mean=7.9, standard deviation (SD)=4.12), 2 for leech iDNA (mean=2.6, SD=2.65), and 21 for water eDNA (mean=22.62, SD=5.16), indicating substantial differences in the detection efficiency of the three methods.

Camera traps detected a total of 32 wild terrestrial vertebrate species (Figure 3; Supplementary Figure S2), representing 31.4% (32/102) of total species. These included 11 species from Carnivora, 4 from Cetartiodactyla, 3 from Primates, 6 from Rodentia, one from Scandentia, and 7 from Galliformes. 11 species (10.8% of the total) were detected exclusively by camera traps.

Leech iDNA also detected 32 wild terrestrial vertebrate species (31.4% of the total), including 5 from Carnivora, 3 from Cetartiodactyla, 2 from Eulipotyphla, 4 from Primates, 6 from Rodentia, one from Scandentia, 4 from Galliformes, 3 from Passeriformes, and 2 amphibians from Anura and 2 from Caudata. 12 species (11.8% of the total) were unique to leech iDNA. Among these detections, 26 species were identified using the 12S marker, 23 species were identified using the 16S marker, 17 species were detected by both genes, 9 species were detected by 12S only, and 6 species were detected by 16S only (Supplementary Figure S3).

Water eDNA detected 71 wild terrestrial vertebrate species (69.6% of the total), including 4 species from Carnivora, one from Cetartiodactyla, 6 from Chiroptera, 6 from Eulipotyphla, 2 from Primates, 22 from Rodentia, one from Scandentia, one from Columbiformes, one from Cuculiformes, 3 from Gruiformes, 9 from Passeriformes, one from Piciformes, one from Psittaciformes, and 10 amphibians from Anura and 2 from Caudata. 54 species (52.9% of the total) were detected exclusively by water eDNA. Of the species identified by water eDNA, 53 were detected using the 12S gene and 38 using the 16S gene, with 20 species detected by both genes, 33 species detected by 12S only, and 18 species detected by 16S only (Supplementary Figure S4).

In terms of taxonomic composition, camera traps recorded the highest numbers of species within Carnivora (11 species), Cetartiodactyla (4 species), and Galliformes (7 species). Leech iDNA detected fewer species in these groups, including Carnivora (5 species), Cetartiodactyla (3 species), and Galliformes (4 species), although certain taxa, such as the palm civet (*Paradoxurus hermaphroditus*) and Chinese pheasant (*Syrmaticus humiae*), were detected exclusively by leech iDNA. Water eDNA detected the fewest species in these groups, with 4 in Carnivora, one in Cetartiodactyla, and none in Galliformes. For Primates, leech iDNA detected the highest number of species (4), followed by camera traps (3) and water eDNA (2). The black-headed langur (*Trachypithecus melamera*) was detected exclusively by camera traps. Other groups, such as Chiroptera, were detected only by water eDNA, while Eulipotyphla, Rodentia, non-Galliformes bird species, and amphibians were most commonly detected by water eDNA (Figure 4; Supplementary Figure S2).

DISCUSSION

Understanding species distributions and diversity patterns underpins effective biodiversity conservation and management. Different survey techniques inherently vary in their capacity to detect specific taxa, and a critical evaluation of their relative merits is essential for designing efficient and accurate monitoring programs. This study compared three complementary approaches, camera trapping, leech-derived

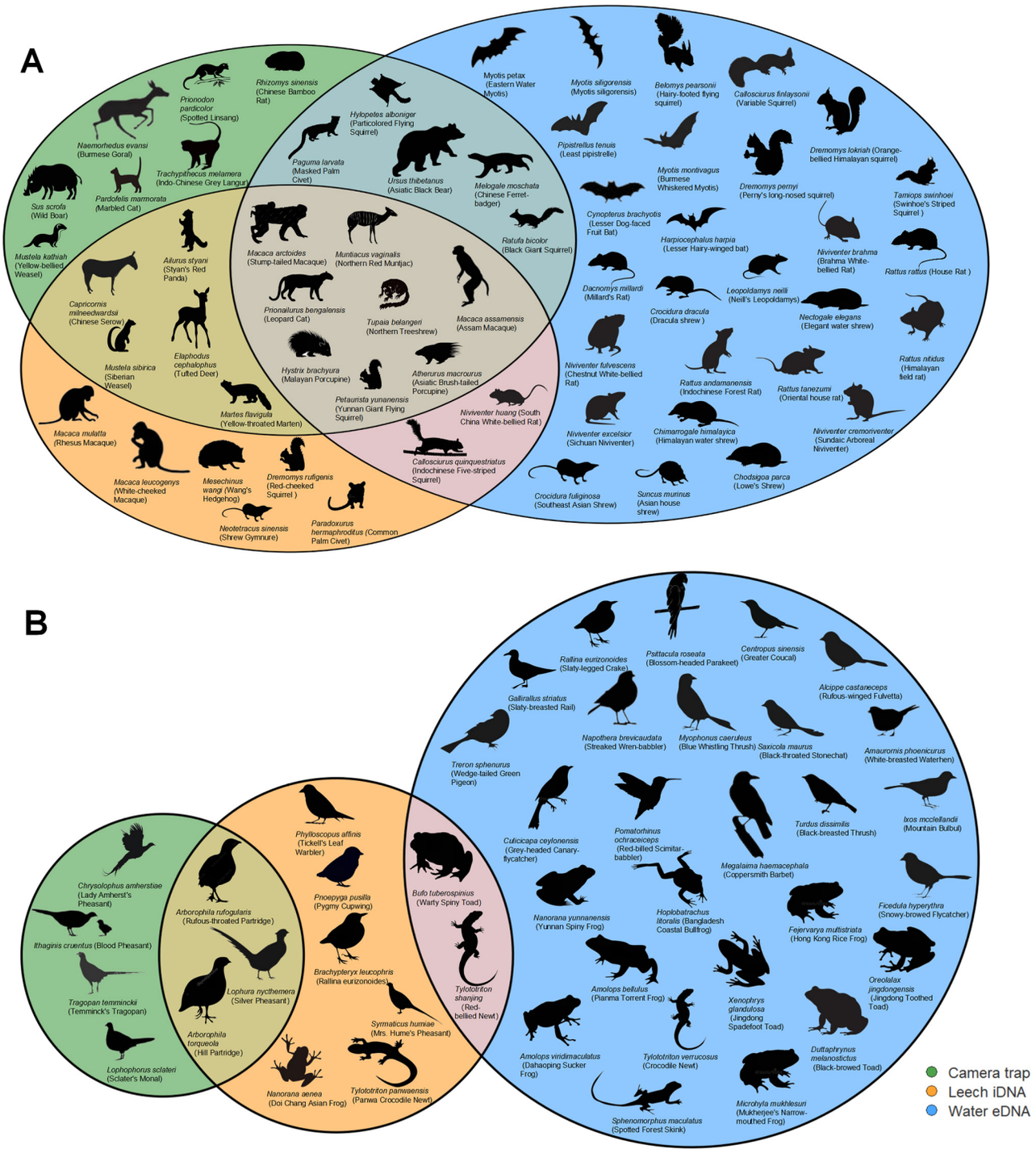

Figure 3 Venn diagrams showing species identified by the three different detection methods and their overlap

Diagram A represents mammals; Diagram B represents birds and reptiles. Circle size is proportional to the number of species detected. Green circle represents species detected by camera traps, orange circle represents species detected by leech iDNA, and blue circle represents species detected by water eDNA. Overlapping areas indicate species detected by two or three methods.

iDNA, and water-derived eDNA, which collectively detected 102 species of wild terrestrial vertebrates. By integrating these methods, our study achieved a higher overall species count and broader taxonomic coverage than any single method alone, underscoring the value of methodological complementarity in biodiversity assessments. However, direct comparisons of detection outcomes must be interpreted with caution, as differences in environmental context, species assemblages, and sampling effort can substantially influence observed patterns (Table 2). Metrics such as species detected per unit of sampling effort provide a more standardized basis for cross-study comparisons than absolute species counts. While all three approaches effectively identified target species, each method captured distinct components of the vertebrate community, reflecting differences in detection sensitivity, spatial coverage, and temporal resolution. Thus, the comparative framework presented here is intended to guide the optimization of monitoring strategies rather than to establish an absolute performance hierarchy. These findings highlight the value of integrating multiple, complementary

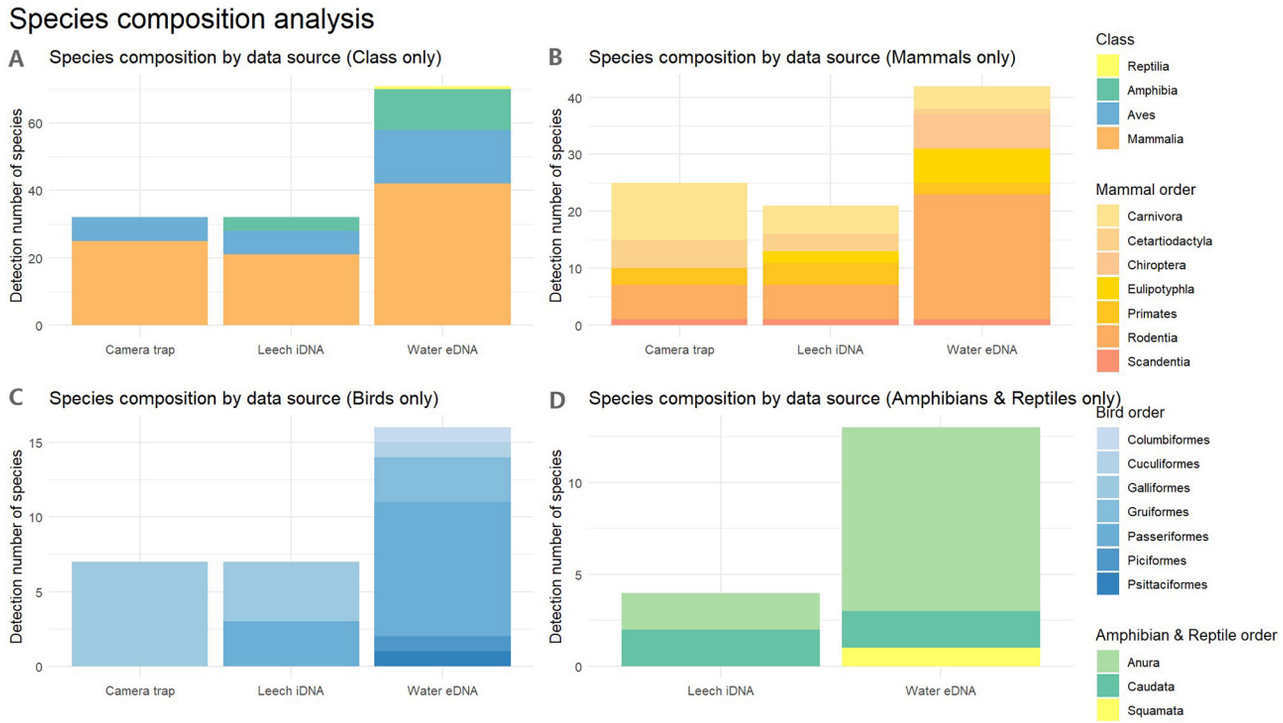

Figure 4 Comparative species composition detected by three monitoring methods (camera trap, leech iDNA, and water eDNA)

A: Composition structure at the class level. B–D: Composition characteristics at the order level for mammals, birds, and amphibians/reptiles. Legend on right indicates the class and order corresponding to each color. Bar height represents number of species detected.

Table 2 Comparative efficiency of biodiversity detection methods across studies

Detection method	Study region	Sampling effort	Taxonomic groups detected (mammals/birds/ amphibians and reptiles)	Detection efficiency	Reference
Live-traps	Andorra	8 points, 8 640 trap nights	13/0/0	0.001 5 species/trap-night	(Torre et al., 2016)
Camera trap	China (Yunnan)	17 areas, 113 204 days	34/0/0	0.000 3 species/trap-night	(Hu et al., 2025)
Camera trap	China (Xizang, Yunnan)	35 areas, 379 200 days	60/15/0	0.000 2 species/camera-day	(Li et al., 2020)
Camera trap	China (Yunnan)	3 areas, 10 400 days	18/44/0	0.006 0 species/camera-day	(Gao et al., 2017)
Camera trap	Vietnam, Laos	140 points, 17 393 days	27/0/0	0.001 6 species/camera-day	(Tilker et al., 2020)
Field survey and interviews	China (Yunnan)	239 lines, 72 person-days	0/0/81	1.125 species/person-day	(Hou et al., 2023)
Leech iDNA	China (Yunnan)	172 points, 30 468 leeches	34/22/30	0.002 8 species/leech	(Ji et al., 2022)
Leech iDNA	Vietnam, Laos	193 samples, 2 043 leeches	25/0/0	0.012 2 species/leech	(Tilker et al., 2020)
Fly iDNA	Australia	2 lines + 1 area, 920 fly	23/1/3	0.029 3 species/fly	(Fernandes et al., 2024)
Camera trap	China (Yunnan)	60 points, 16 041 days	25/7/0	0.002 0 species/camera-day	This study
Leech iDNA	China (Yunnan)	25 points, 847 leeches	28/4/0	0.037 8 species/leech	This study
Water eDNA	China (Yunnan)	5 sampling points, 26 samples (30 L/point)	42/16/13	2.73 species/sample	This study

*Note: Studies differ in spatial scale (point vs area sampling), duration, and taxonomic focus. Efficiency values allow more standardized cross-study comparison than absolute counts.

detection methods to enhance both the efficiency and accuracy of vertebrate biodiversity monitoring.

Advantages and limitations of camera traps

Camera trapping provides a powerful tool for monitoring medium to large-sized mammals and ground-dwelling birds, capturing long-term, continuous data on species presence and activity (Trolliet et al., 2014). Furthermore, the approach provides spatially and temporally explicit data, enabling fine-scale analyses of habitat use and movement patterns, and allowing investigation of how these may vary by reproductive status, season, or environmental conditions. Its ability to document natural behaviors without direct human presence further strengthens its value for ecological studies. However, its reliance on morphological traits for species identification constrains its utility to larger and visually distinctive taxa, resulting in the under-detection of small-bodied or morphologically similar species. Additionally, deployment in remote areas or for extended periods entails considerable logistical and financial costs, including the acquisition, installation, maintenance, and regular replacement of batteries and memory cards. Detection probabilities can also be highly sensitive to seemingly minor variations in camera angle or placement, leading to spatial inconsistencies and taxonomic biases in recorded communities (Burton et al., 2015). Comprehensive coverage therefore requires substantial field

effort and careful standardization. Despite these limitations, camera trapping remains an indispensable approach for large-scale terrestrial vertebrate monitoring, offering considerable advantages in generating high-resolution, long-term datasets for medium- and large-bodied species with relatively favorable cost-effectiveness in extended surveys.

Advantages and limitations of DNA-based approaches

The DNA-based methods —leech iDNA and water eDNA— collectively detected 91 species, including 70 species not recorded by camera traps. Conversely, 11 species were detected exclusively by camera traps and were absent from both leech iDNA datasets. A well-recognized limitation of DNA-based monitoring lies in the incompleteness of reference sequence databases, particularly in biodiverse regions lacking comprehensive baseline surveys (Beng & Corlett, 2020). In this study, however, all species detected by camera traps had corresponding reference sequences, indicating that their absence from DNA detections was not due to a lack of reference data. Sequence alignment further revealed that most camera-detected species either matched primers exactly or differed by only a single base at the 5′ end, suggesting that primer mismatches were not the primary cause of non-detections in DNA datasets. Analysis of camera trap records (Supplementary Figure S1) demonstrated that these undetected species generally exhibited low detection probabilities, consistent with small or sparsely distributed populations, thereby reducing their likelihood of capture by leech sampling. For example, species such as *Macaca mulatta* and *T. melamera* (Primates), *Mustela sibirica* and *Prionodon pardicolor* (Carnivora), and *Tamiops swinhoei* and *A. macrourus* (Rodentia) were detected with low probabilities, as indicated by their positions in the lower left corner of Supplementary Figure S1. Moreover, spatial overlap between camera trap locations and water eDNA sampling sites was limited, as the latter were selected to represent diverse aquatic habitats across the study area, whereas camera traps were deployed in areas with higher potential for terrestrial vertebrate activity. Water eDNA sampling was also incomplete, with many potential water bodies and sampling points unsampled, indicating that detection saturation had not been reached. This spatial and temporal mismatch, coupled with the incomplete saturation of eDNA sampling effort, likely contributed to the absence of certain camera-detected species from the water eDNA dataset.

With expanded eDNA sampling coverage and increased collection frequency, the detection of additional medium- to large-sized vertebrates is expected. However, accurately distinguishing closely related species —particularly domestic species and their wild counterparts (e.g., domestic pigs vs. wild boars, dogs vs. wolves) —remains challenging when relying on the short mitochondrial DNA fragments commonly employed in current metabarcoding protocols. Achieving reliable resolution in such cases may require the use of species-specific or even population-specific primers. This limitation underscores the difficulty DNA-based methods face when monitoring sympatric, closely related species. Camera traps, although independent of genetic markers, encounter analogous challenges when morphological similarity or behavioral convergence reduces the reliability of visual identification. Each approach thus presents distinct strengths and weaknesses, and methodological choice should be informed by the specific ecological and taxonomic context.

DNA-based techniques are particularly well suited to detecting smaller-bodied species (Beng & Corlett, 2020), a pattern supported by the present study (Supplementary Figure S5). Both leech iDNA and water eDNA yielded higher detection rates for small mammals, birds, amphibians, and reptiles (Figure 4; Supplementary Figure S2,S5), taxa that are generally underrepresented in conventional surveys. Compared to leech iDNA, water eDNA detected a broader range of flying species (e.g., bats, cranes, sparrows), aquatic amphibians, and a greater diversity of rodents and insectivores. Although leech-derived detections were fewer overall, several species were recorded exclusively through this method, indicating that leech iDNA and water eDNA function in a complementary manner, each enhancing the overall scope of biodiversity detection.

Water eDNA, despite involving the fewest sampling points, produced the highest species richness in this study. This efficiency likely reflects the transport of eDNA by the natural flow of water, integrating biological signals from upstream habitats and enabling broader taxonomic coverage from limited sampling effort. Nevertheless, achieving more exhaustive biodiversity inventories —especially of elusive or low-abundance taxa such as medium to large-bodied vertebrates —would benefit from strategically expanding sampling sites and replicates within hydrologically accessible areas. The principal advantage of water eDNA lies in its ability to efficiently and cost-effectively capture species information across both aquatic and adjacent terrestrial environments. However, this advantage is accompanied by reduced spatial resolution in lotic systems, making it difficult to detect fine-scale differences in species distribution. In this study, flowing water eDNA revealed diverse species but could not pinpoint their exact upstream locations. In contrast, lentic water bodies such as ponds or wetlands are more likely to yield localized species signals and thus higher spatial resolution of species presence. The placement of sampling points is further constrained by the physical configuration of drainage networks. While some field studies, such as those conducted in African savannas, have used artificial water sources for eDNA sampling (Farrell et al., 2022), such methods are less feasible in remote montane forest ecosystems. Recent developments in hydrodynamic modeling offer potential for refining the spatial attribution of riverine eDNA detections (Carraro et al., 2023), which may enhance the interpretability of such datasets in future applications.

When financial resources are limited, optimizing biodiversity monitoring requires a strategic balance between the number of sampling sites and the intensity of sampling at each site, guided by the specific objectives of the study. For large-scale biodiversity assessments and mapping of species distributions, prioritizing more sampling sites generally provides greater spatial representativeness (Altermatt et al., 2023). Conversely, when the goal is to detect rare or elusive species, increasing sampling effort at selected sites may enhance detection probability (Chacko et al., 2023). Within the Gaoligong Mountains, an optimal approach would first maximize spatial coverage to establish a comprehensive baseline, followed by intensified sampling at key locations to refine detection of elusive species in subsequent monitoring phases.

Water eDNA collection, while highly effective, requires specialized technical expertise and equipment such as electric pumps and filtration systems, which increase both operational

complexity and cost. These challenges are amplified in remote mountainous areas, where additional technical support and equipment transport further elevate operational costs. Nonetheless, for surveys spanning extensive geographic areas, water eDNA remains comparatively cost-efficient, as its capacity to integrate biodiversity signals over large areas reduces the number of sampling points needed and lowers the monitoring cost per unit area.

In this study, leech iDNA yielded a total species count comparable to that of camera trapping; however, the cost per species detected was higher for leech sampling. Here, cost per species refers to the total expenditure for each method divided by the number of species it detected. The elevated cost for leech iDNA reflects its reliance on random encounters between leeches and host species, with detection efficiency constrained by encounter probability and the number of leeches collected per sample. Increasing the number of leeches processed from each site could potentially enhance detection rates without proportionally increasing costs, thereby reducing the per-species monitoring cost. Unlike riverine water eDNA, which naturally accumulates genetic material from multiple upstream sources, leech iDNA captures are more strongly influenced by host behavior and abundance. Leeches are particularly effective at capturing DNA from ground-active species; for instance, a study in the Ailao Mountains successfully detected all known ground-dwelling bird species in the region using leech iDNA (Ji et al., 2022). In this study, leech iDNA detections aligned closely with species frequently captured by cameras, although detection rates were lower for species with infrequent camera records. A key advantage of leech iDNA monitoring is its lower threshold for sampling, requiring minimal equipment and only basic training for field staff. This simplicity enables non-specialists to collect samples during routine patrols. Consequently, leech iDNA represents a highly adaptable approach for biodiversity monitoring in tropical and subtropical regions (Fahmy et al., 2023).

Necessity of using multiple metabarcodes

Analysis of both leech iDNA and water eDNA revealed substantial overlap in species identified by the 12S and 16S gene markers, as well as a number of unique detections (Supplementary Figure S4). The broader taxonomic breadth of the 12S marker, which targets all vertebrates, likely explains its higher species yield, whereas the mammal-optimized 16S marker provided more focused amplification, although still detected taxa outside its primary target group. These findings, consistent with earlier studies demonstrating the complementary nature of different genetic markers (Kress et al., 2015), show that combining multiple barcodes yields more comprehensive biodiversity inventories and should be considered standard practice in environmental DNA-based surveys.

In terms of sequencing efficiency, the 16S marker produced a higher proportion of usable reads than 12S, likely due to its more specific primer design, which reduces non-target amplification and minimizes data loss. Across sampling approaches, leech iDNA achieved greater data utilization than water eDNA. This disparity may be attributed to the open and heterogeneous nature of aquatic environments, where water samples often contain large amounts of non-target DNA. In this study, human DNA contamination accounted for 7.33% of 12S and 23.56% of 16S water eDNA reads, substantially higher than in leech iDNA samples (Table 1). Additionally, fish DNA dominated water eDNA, comprising over half of total 12S reads, although its proportion was markedly lower in upstream river regions. This spatial variation highlights the importance of strategic site selection: sampling from upstream locations can reduce fish DNA interference, while avoiding downstream sites near human settlements can minimize contamination from domestic animals. Where non-target DNA remains high, increased sequencing depth can help recover target taxa. Collectively, these results emphasize that marker choice, environmental context, and spatial design are critical determinants of detection efficiency in eDNA-based biodiversity monitoring.

CONCLUSION

The results of this study demonstrate that no single monitoring approach can adequately capture the full spectrum of species diversity or accurately characterize community structure. An integrative framework that combines complementary techniques can effectively bridge this gap, yielding a more holistic and accurate representation of species data. Camera trapping provides spatially and temporally explicit insights into species distribution and activity patterns; leech iDNA supplements detection of terrestrial species, particularly elusive species; and water eDNA extends coverage to aquatic organisms and upstream areas. When deployed in concert, these methods yield a more robust and comprehensive inventory, a benefit that is especially critical in species-rich and structurally complex ecosystems.

Technological advancements are expected to further strengthen such integrative monitoring. For instance, artificial intelligence-based automated image recognition coupled with high-throughput DNA sequencing will accelerate and refine data processing. The integration of remote sensing technologies and environmental sound monitoring will expand the scope of ecological monitoring, especially in remote areas such as mountainous regions and dense forests.

Considering method-specific strengths, limitations, cost structures, and operational feasibility, our evidence supports a strategic, multi-method approach to biodiversity monitoring. This is particularly important in heterogeneous landscapes like the Gaoligong Mountains, where the limitations of any one method can be offset by the strengths of others. Future priorities should focus on optimizing method combinations, refining technical processes, and reducing costs to maximize detection sensitivity, spatial coverage, and long-term sustainability of monitoring programs.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online. The data supporting the findings of this study have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA1304758.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS CONTRIBUTIONS

C.Y.W. wrote the article, collected water samples, and performed molecular experiments and data analysis. J.H.L. collected leech samples and wrote part of the original article. R.B. collected camera samples and conducted data processing and bioinformatics analysis. Y.Q.J. performed statistical analyses and provided thesis guidance. Y.M.R.C., S.J.Z., J.T.D., J.Y.T., and C.J.Y. collected leech samples and contributed to experimental design. X.Y.L. collected camera samples, performed data processing, and provided guidance for the study and article writing. All authors read and approved the final version of the manuscript.

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