Evaluation of nanopore sequencing for increasing accessibility of eDNA studies in biodiverse countries

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Abstract

Biodiversity loss is a global challenge of the 21st century. Environmental DNA (eDNA)-based metabarcoding offers a cost- and time-efficient alternative to conventional biodiversity surveys, enabling detection of rare, cryptic, and elusive species from environmental samples. However, limited access to genomic technologies restricts the application of eDNA metabarcoding in highly biodiverse remote regions and low- and middle-income countries (LMICs). Here, we directly compared the latest portable nanopore sequencing methods with established Illumina sequencing for vertebrate eDNA metabarcoding of Zambian water samples. Our results show that due to recent improvements in sequencing chemistry and

optimized basecalling, nanopore sequencing data can recapitulate or even surpass established protocols, demonstrating the feasibility of *in situ* biodiversity assessments. eDNA- and camera trap-based species detections had minimal overlap in species detections, suggesting a complementary rather than substituting application of these biodiversity monitoring technologies. We finally demonstrate that our entire eDNA workflow can be successfully implemented in a mobile laboratory under remote field conditions by completing all steps—from sample collection to data analysis—within the Luambe National Park in Zambia. This approach has important implications for capacity building in LMICs and for overcoming limitations associated with sample export.

Keywords

eDNA, portable sequencing, vertebrates, Zambia, metabarcoding

37 **Introduction**

38 Biodiversity loss is one of the major challenges of the 21st century [1], but efforts to prevent
39 biodiversity loss are often hindered by a lack of knowledge of species distributions and
40 dynamics [2,3]. Environmental DNA (eDNA) approaches can provide a cost- and time-efficient
41 alternative to conventional biodiversity monitoring methods by detecting taxa, including rare,
42 cryptic, and elusive species, from metabarcoding their genetic material in environmental
43 samples such as water, soil, or air, and subsequent amplicon sequencing [4-6]. Such eDNA
44 approaches have recently become cheaper and more reliable in monitoring the temporal and
45 spatial distribution and dynamics of species, populations, and communities [7][8][9]. The lack
46 of access to genomic technology, however, hinders the application of metabarcoding
47 approaches in remote areas and low- and middle-income countries (LMICs), which often
48 contain the globally largest amount of biodiversity [10-12]. Simultaneously, international
49 treaties such as the Convention on International Trade in Endangered Species of Wild Fauna
50 and Flora [13] and data sovereignty principles as specified by the Nagoya Protocol on Access
51 and Benefit Sharing [14] and the Global Indigenous Data Alliance [15] often require the
52 generation and usage of such genomic data close to the species' origin.

53 Long-read nanopore sequencing technology as developed by Oxford Nanopore Technologies
54 (ONT) is the first genomic technology that enables rapid *in situ* sequencing at low upfront
55 investment costs through its portable devices. This technology therefore holds the promise of
56 putting eDNA research in line with international treaties and data sovereignty principles, while
57 saving time and resources otherwise spent on sample transport and exportation, shortening
58 the gap between research and decision-making and potentially empowering local researchers,
59 conservationists, and decision-makers [11,16]. First applications of nanopore sequencing to
60 metabarcoding in general [17-19] and eDNA specifically [20,21] have shown promising results
61 in species but in comparison to the established sequencing platform for metabarcoding, short-
62 read Illumina sequencing, remained affected by the relatively high sequencing error rates.
63 Bludau *et al.* have, however, also shown that this lack in sequencing accuracy can be

compensated by nanopore sequencing's long-read capability, which enabled species-level resolution in their protistan studies by substantially increasing the targeted amplicon size. While the error rate of this previously applied nanopore sequencing chemistry ("R9" chemistry) was estimated at anywhere between 5% and 22% [17,19], recent substantial improvements in the nanopore design ("R10" chemistry) and basecalling algorithms now allow for highly accurate nanopore read- and consensus-level accuracy. Doorenspleet *et al.* were the first to use these accuracy improvements to apply nanopore-based eDNA to successfully monitor marine vertebrates from aquarium and sea water. Similarly, Bludau *et al*. showed that an increase of the targeted amplicon size also improves species detection when using the latest nanopore chemistry; however, fragmentation of the marine vertebrate's DNA in real-world environments such as the sea inhibits the amplification of long stretches of DNA [22].

Here, we conducted an eDNA study to directly compare the latest improvements in portable nanopore sequencing with established Illumina sequencing as well as camera trapping for monitoring terrestrial vertebrate diversity from freshwater samples in Zambia. We additionally tested the *in situ* implementation of eDNA methods in a mobile laboratory setting by applying our complete eDNA workflow from water collection to data analysis on site in Luambe National Park, Zambia. Zambia serves as a good example of a biodiversity-rich LMIC that shares borders and ecosystems with eight other African countries and is estimated to be home to 242 mammals, 757 bird, 74 amphibian and 156 reptiles [23]. Zambia has further been described as "a large patchwork of important ecosystems" [25]., with its subtropical savanna ecosystems being characterized by extreme oscillations of precipitation and water availability during the wet and dry seasons [24,25]. This seasonality results in an increased reliance on mobility for both humans and animals to access water [25] and therefore imposes challenges for monitoring highly mobile vertebrates across large areas. In arid and semi-arid systems where surface water is often scarce, natural and artificial water holes further act as gathering sites for mammals and birds [26], making them potentially optimal sources of eDNA for monitoring vertebrate fauna [27-29].

Materials and Methods

Environmental DNA collection

For sequencing comparisons, we took water samples at five locations in the Luambe and Lukusuzi National Parks within the Luangwa Valley in Eastern Zambia between July 13th and 16th, 2023 (Fig A-F). In Luambe National Park, three water bodies were sampled: an artificial pond near an established tourism facility (CAM: 12.45880° S, 32.14662° E) (Fig 1B) and two permanent natural ponds (P2: 12.48503° S, 32.17606° E; P3: 12.48246° S, 32.19048° E) (Fig 1C-D). In Lukusuzi National Park, samples were collected from two sites along the Lukusuzi River (L1: 12.71192° S, 32.50254° E; L2: 12.71247° S, 32.50226° E) (Fig 1E-F). These locations were chosen because previous camera trap-based monitoring had identified them as hotspots of vertebrate activity (Fig 1A-F). At each water body, three biological replicates (A, B, and C) were collected. For the *in situ* application (see "*In situ* eDNA application"), freshwater samples were collected at three sites of the Mwendi Lagoon (Mw: 12.53318° S, 32.1435° E) in Luambe National Park between June 11th and 16th, 2025 (Fig 1G).

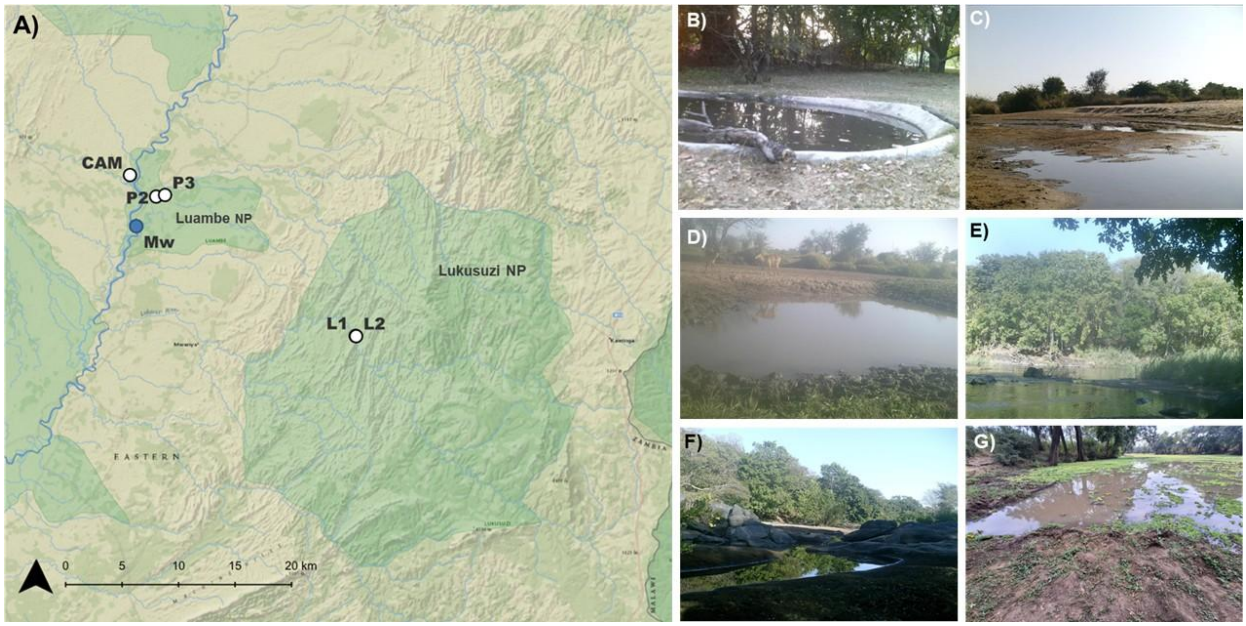

Fig 1. Map and pictures of study sites. A) Map showing the sampling locations within Luambe and Lukusuzi National Parks (Mw: blue dot) ; B) CAM: Artificial pond in Luambe

108 Camp; C) P2: Natural waterhole 1 in Luambe NP; D) P3: Natural waterhole 2 in Luambe NP;
109 E) L1: Lukuzusi backwater site 1; F) L2: Lukuzusi backwater site 2; G) Mw: Mwendi Lagoon
110 (sampling site for the *in situ* application). Basemap: Esri, National Geographic Style (accessed
111 September 2025) via QGIS.

112 We collected freshwater samples by using sterile plastic scoops and storing them in sterile
113 Whirl-Pak® bags (Thermo Scientific, Germany) for transport to the camp. At the camp, one liter
114 of water per sample was filtered using 250ml cups with 0.45 µm nitrocellulose filters (Thermo
115 Scientific Nalgene, Germany) and a Vampire Vacuum Pump (Bürkle, Germany) to speed up
116 the process. For samples with high sediment content, multiple nitrocellulose filters were
117 required to filter the entire liter due to clogging; for these samples, final DNA extracts were
118 subsequently pooled across filters per replicate. After filtration, the nitrocellulose filters were
119 first stored in sterile kraft bags and then placed in sterile ziplock bags with silica gel beads.
120 The samples were frozen at -20°C before and after transportation from Zambia to Germany.
121 For the sequencing comparisons, all molecular work was subsequently conducted at the
122 Helmholtz Center Munich laboratories in Germany under the corresponding research permits
123 from the Zambian Department of National Parks and Wildlife (Reference number:
124 NPW/8/27/1).

125 **Environmental DNA extraction**

126 The nitrocellulose filters were cut in half using a sterile scalpel, sterile gloves, and a FFP2
127 mask, then stored separately in 1.5 ml Eppendorf tubes at -20°C until further processing; half
128 of a filter was then used for DNA extraction while the other half was kept as a backup. DNA
129 was extracted from all samples using the DNeasy PowerSoil Kit (Qiagen) following the
130 manufacturer's instructions, with the exception that DNA was eluted in only 50 µl of elution
131 buffer in the final step.

132 For the filter negative control, one liter of sterile Milli-Q® water was filtered. Together with all
133 biological replicates across all sampling sites, this resulted in 16 DNA extracts, whose DNA

134 concentrations were measured with an Invitrogen™ Qubit™ 3.0 Fluorometer using the High
135 Sensitivity (HS) Assay.

136 **PCR amplification and metabarcoding**

137 Metabarcoding was performed using two mitochondrial primer sets. The vertebrate-specific
138 12S rRNA primer pair 12SV05 (forward 5′-TTAGATACCCC ACTATGC-3′; reverse 5′-
139 TAGAACAGGCT CCTCTAG-3′) amplifies a ~97 bp fragment of the 12S rRNA gene [30].
140 Mammals were further targeted with the 16S rRNA primer pair 16Smam1 (forward 5′-
141 CGGTTGGGGTGACCTCGGA-3′) and 16Smam2 (reverse 5′-
142 GCTGTTATCCCTAGGGTAACT-3′), which amplifies a ~95 bp fragment of the 16S rRNA gene
143 [31].

144 Each sample extract was amplified in PCR triplicates, and a filter negative control in two PCR
145 replicates, plus one PCR control, resulting in overall 48 PCR products. The PCR reactions
146 were set up in 25 µL volumes consisting of 2 µL DNA template, 12.5 µL Phusion® High-Fidelity
147 PCR Master Mix, 1.25 µL of each primer, and 8 µL of DNase-free water. For the 12S rRNA
148 primer pair the cycling parameters were as follows: 95°C for 10 minutes, followed by 40 cycles
149 of 94°C for 30 s, 60°C for 30 s, and 70°C for 60 s, with a final extension at 72°C for 7 min. For
150 the 16S rRNA primer pair, the cycling parameters were as follows: 95°C for 10 min, followed
151 by 40 cycles of 95°C for 12 s, 59°C for 30 s and 70°C for 25 s, with a final extension at 72 °C
152 for 7 min.

153 A symmetrical tagged primer approach was used to multiplex the samples during amplification.
154 Unique 9 bp tags, generated using Barcode Generator
155 (https://github.com/lcomai/barcode_generator), were added to the 5′ ends of both the forward
156 and reverse primers for each primer set. This tag length has been shown to be suitable for
157 multiplexing amplicons for nanopore sequencing [32]. All tags were designed to differ by at
158 least 3 bp from each other, and the same tag was used on both the forward and reverse
159 primers of each PCR product.

160 Following amplification, one replicate PCR product was respectively visualized on 2% agarose
161 gels with GelRed and Invitrogen™ 100 bp DNA. Amplification was confirmed by the presence
162 of a band of the expected size, which was the case for all visualized samples (including the
163 negative controls).

164 **Sequencing library preparation and sequencing**

165 The 48 PCR products were pooled by taking 10 µl of each product and then cleaned using a
166 2:1 bead-to-DNA ratio with AMPure XP Beads (Beckman Coulter, Germany), followed by
167 resuspension in 100 µL of DNase-free water. This final pool was separated into two 50 µL
168 volumes as input for nanopore and Illumina sequencing, respectively.

169 Nanopore sequencing was performed using the portable MinION Mk1B device. Library
170 preparation was done using the Ligation Sequencing Kit (SQK-LSK114), following the
171 manufacturer's Ligation Sequencing Kit Amplicons protocol with the following modification: At
172 the end of the protocol, 30 µL of elution buffer was used instead of the specified 15 µL. This
173 was done to simultaneously prepare two libraries, which was possible due to high DNA input
174 into the library preparation. Both libraries were consecutively sequenced for 16 hours on one
175 flow cell, separated by a flow cell wash step.

176 Illumina sequencing was performed by Novogene, GmbH, Germany, using their 2x150 bp
177 protocol on a NovaSeq 6000.

178 **Data processing and OTU clustering**

179 Nanopore raw data was basecalled using Dorado's (v1.0.2) super high accuracy model (SUP
180 v5.0.0; https://github.com/nanoporetech/dorado). The resulting FASTQ files were
181 demultiplexed using OBITools4's obimultiplex command, allowing for a maximum of two errors
182 in the tags (https://git.metabarcoding.org/obitools/obitools4). Primers were trimmed using
183 Cutadapt (v4.9) [33]. Subsequent Operational Taxonomic Unit (OTU) processing steps were
184 performed using VSEARCH (v2.28.1) [33]: We first filtered the reads by setting the VSEARCH

185 fastq_maxee parameter to thresholds of 1.0: For a 97 bp sequence, a fastq_maxee value of
186 1.0 corresponds to average per-base quality scores of approximately 20 using the formula: Q
187 = $-10 \times \log_{10}(\text{fastq_maxee} \div L)$, where Q is the average per-base Phred quality score and L is
188 the read length. Therefore, lower fastq_maxee thresholds require higher average per-base
189 quality scores to keep the expected error per read below the specified limit. The next steps
190 included dereplication (with singleton removal), chimera removal, and clustering at a 99%
191 sequence identity threshold; the final OTUs were pooled across respective PCR replicates.

192 Illumina paired-end data were first merged and then demultiplexed with Obitools 4 allowing for
193 a maximum of two errors in the tags. Primers were trimmed using Cutadapt (v4.9) [33].
194 Subsequent OTU processing steps were identical to the OTU processing steps of the
195 nanopore sequencing data.

196 **Taxonomic assignments**

197 Amphibian, bird, mammalian, and bird species lists reported in Zambia were obtained from
198 the SASCAL portal
199 (http://data.sasscal.org/metadata/view.php?view=doc_documents&id=3154, 2017). This full
200 species list (S1 Table) was used as a list of regional species, and to assess our study's
201 database incompleteness database (S2 Table).

202 Taxonomic assignment of the OTUs was done using the global alignment function
203 usearch_global VSEARCH (v2.28.1) [34] against the Midori2 databases Unique Nucleotide
204 short ribosomal RNA version 266 (MIDORI2_UNIQ_NUC_GB266_srRNA_BLAST) and the
205 Unique Nucleotide long ribosomal RNA version 266
206 (MIDORI2_UNIQ_NUC_GB266_lrRNA_BLAST), which contain curated non-redundant 12S
207 and 16S rRNA sequences, respectively [35]. Only the best taxonomic hits with at least 90%
208 sequence similarity score and 80% query coverage score were taken and then filtered for local
209 taxa detections based on the list of regional species: Assignments with ≥98% identity to a
210 reference sequence from Zambia were considered reliable species-level identifications:

211 matches between 95–98% identity were assigned at the genus level, while those between 90–
212 95% identity were conservatively classified at the family level. We only focused our
213 subsequent analyses on primary non-terrestrial vertebrate classes. Taxa detected in the
214 negative controls were removed from the data.

215 **Camera Trapping**

216 One week prior to eDNA sampling, camera traps (SECACAM Wild-Vision Full HD 5.0) were
217 put up at sampling locations to validate species detections. Cameras, equipped with passive
218 infrared sensors, were set to picture-mode and recorded for 24 hours. The number of deployed
219 camera traps varied from 2 to 5 cameras across sites, depending on the size and accessibility
220 of the water bodies. Cameras were positioned to cover the entire waterbodies with priority on
221 established drinking places by large mammals. Drinking places were determined by visual
222 assessments on mammal tracks, scat, or further signs of presence.

223 ***In situ* eDNA application**

224 We finally conducted our entire protocol including sample collection, molecular work, and
225 bioinformatic analyses on site in the Nsanga Research Camp within Luambe National Park.
226 Freshwater samples were collected daily from three sites (A–C) spaced ~30 m apart at the
227 Mwendi Lagoon (Mw: 12.53318° S, 32.1435° E) in Luambe National Park from June 11th to
228 16th. For the molecular work, we used a mobile laboratory setting assembled within a camping
229 tent (S4 Appendix). For the bioinformatic analyses, we used a high-performing laptop (Razer
230 Blade 15.6″ with 16 GB RAM, an NVIDIA RTX 4070 GPU, and an Intel Core i7 processor). We
231 again used camera trapping in parallel (see “Camera Trapping”), with one camera per
232 sampling site and daily assessments from sampling day 2.

233 We implemented the following modifications of our nanopore sequencing-based eDNA
234 protocol for the *in situ* approach:

235 1) To reduce contamination risk, the following protocol steps were modified: Instead of
236 using 0.45 µm nitrocellulose filters and centralized processing by a vacuum pump in
237 the camp, Smith and Root™ eDNA Filter Packs were used to filter three liters of
238 wateron site while minimizing handling. We used PES (Polyethersulfone) filters with a
239 larger pore size (1.2 µm) to reduce clogging when filtering larger volumes of water.
240 With this approach it took between 15 min and 25 minutes to filter 3L of water. The
241 filters were stored in sterile bags and stored at -20ºC until DNA extraction.
242 2) We used the DNeasyPowerWater Kit (Qiagen) for DNA extraction following the
243 manufacturer's instructions, with the exception that DNA was eluted in 50 µl of elution
244 buffer in the final step. We used this kit instead of the DNeasy PowerSoil Kit (Qiagen)
245 since it allows to process the entire filter in a faster manner. For the filter negative
246 controls, we used blank filters instead of filtering sterile Milli-Q® water due to lack of
247 access to such volumes of sterile water. We used one blank filter for each of the three
248 DNA extractions of six samples. This resulted in 21 DNA extracts, whose DNA
249 concentrations were measured with an onsite Invitrogen™ Qubit™ 3.0 Fluorometer
250 using the High Sensitivity (HS) Assay.
251 3) We incorporated human blockers in the PCR reaction to reduce human contamination.
252 The PCR reactions changed but the same symmetrical tagging approach as described
253 previously was used for sample multiplexing. Specifically, the PCR reactions were set
254 up in 20 µL volumes consisting of 3 µL DNA template, 0.75 U AmpliTaq Gold, 1× Gold
255 PCR Buffer and 2.5 mM $MgCl_2$ (all reagents from Applied Biosystems); 0.6 µM each of
256 5′ nucleotide tagged forward and reverse primer; 0.2 mM dNTP mix (Invitrogen);
257 0.5 mg/mL bovine serum albumin (BSA, Bio Labs), and 3 µM human blocker (5′–3′
258 TACCCCACTATGCTTAGCCCTAAACCTCAACAGTTAAATC–spacerC3 for the 12S
259 rRNA primers [36] and 5′–3′ GCGACCTCGGAGCAGAACCC–spacerC3 for the 16S
260 rRNA primerss [37]). For the 12S rRNA PCR protocol, the number of cycles was
261 increased from 40 to 45 cycles, and instead of the 60°C we used 59°C. For the 16S
262 rRNA PCR protocol, instead of the 59 °C we used 51 °C. We confirmed amplification

263 using 2% EX pre-cast agarose gels run with the E-Gel™ Power Snap Electrophoresis
264 (Thermo Fischer Scientific). For the PCR pool clean-up, we further changed the
265 bead:sample ratio from 2:1 to 1.5:1 to reduce primer-dimmer carryover.
266 4) Nanopore sequencing was performed using the portable MinION Mk1D device with
267 better temperature control than the Mk1B device.

268 **Results**

269 Read counts varied widely across samples for both sequencing technologies and primer set.
270 For Illumina 12S samples ranged from 3 to 161,352 reads per sample (mean = 48,459; SD =
271 41,615). For Illumina 16S yields ranged from 448 to 470,489 (mean = 121,653; SD = 89,537).
272 For Nanopore 12S yields spanned 18 to 2,424,297 (mean = 273,710; SD = 543,585). For
273 Nanopore 16S yields ranged from 2,029 to 1,433,579 (mean = 227,434; SD = 309,771).

274 Our database revision revealed that the availability of genetic references for Zambian
275 biodiversity in the NCBI repository was limited across all primary terrestrial vertebrate classes
276 58.14% of occurring amphibian species were covered by 12S rRNA entries, 68.60% by 16S
277 rRNA, but only 6.98% by full mitochondrial references. 42.78% of reptile species were covered
278 by 12S rRNA entries, 64.43% by 16S rRNA, but only 3.09% by full mitochondrial references.
279 28.38% of avian species were covered by 12S rRNA entries, 16.43% by 16S rRNA, and
280 15.64% by full mitochondrial references. Finally, mammals were relatively well represented
281 with 58.80% (12S rRNA), 53.60% (16S rRNA) and 57.60% (full mitochondria), respectively.

282 All four primary terrestrial vertebrate classes were detected by nanopore and Illumina
283 sequencing; as *Xenopus* sp. was detected by negative controls, it was excluded from
284 downstream analyses. For both sequencing technologies the highest number of local species
285 detections occurred for mammals (17 and 11 species, respectively; Fig 2). Altogether, 18 taxa
286 were detected by both sequencing technologies while seven taxa were only detected by
287 nanopore, and one taxon only by Illumina sequencing (Fig 2). The two primer sets showed
288 little overlap in amplified taxa, with the 12s rRNA primer set yielding 21 taxa and the 16s rRNA

289 primers 9 taxa, with only four of them in common. Most taxa were detected at the CAM site
290 (n=15) followed by P3 and L1 (n=7, respectively), and L2 (n=6) and P2 (n=5). While most sites
291 showed little overlap in detected taxa, a few common species like *Hippopotamus amphibius*
292 *(*Hippopotamus*)*, *Chlorocebus pygerythrus* (Vervet monkey) and *Papio cynocephalus* (Yellow
293 baboon) were detected at nearly all sites (Fig 2).

Taxonomic ranks				Sampling sites				
Class	Order	Family	Species	CAM	P2	P3	L1	L2
Amphibians	Anura	Ptychadenidae	***Ptychadena anchietae***	▲				
Birds	Charadriiformes	Charadriidae	***Vanellus armatus***		●			
	Anseriformes	Anatidae	***Anas sp.***					●
	Bucerotiformes	Bucorvidae	**Bucorvidae sp.**	●				
	Cuculiformes	Cuculidae	**Cuculidae sp.**	●				
	Accipitriformes	Accipitridae	**Accipitridae sp.**	●				
	Psittaciformes	Psittacidae	***Poicephalus sp.***	●				
	Anseriformes	Anatidae	***Sarkidiornis melanotos***	▲				
Mammals	Artiodactyla	Hippopotamidae	***Hippopotamus amphibius***	●▲	●▲	●▲	●▲	●▲
	Artiodactyla	Bovidae	***Syncerus caffer***		▲			
	Artiodactyla	Bovidae	***Tragelaphus sp.***	●		●		●
	Artiodactyla	Bovidae	***Tragelaphus scriptus***			▲		
	Carnivora	Herpestidae	***Helogale parvula***			●▲	●▲	
	Carnivora	Felidae	**Felidae sp.**	●		●	●	
	Carnivora	Herpestidae	***Herpestes sp.***				●	
	Carnivora	Viverridae	***Viverridae sp.***				●	
	Chiroptera	Vespertilionidae	***Afronycteris nanus***	●▲				
	Chiroptera	Vespertilionidae	***Vespertilionidae sp.***	▲				
	Chiroptera	Rhinolophidae	***Rhinolophus sp.***	●		●		
	Primates	Cercopithecidae	***Chlorocebus pygerythrus***	●▲	●▲	●▲	●▲	
	Primates	Cercopithecidae	***Papio cynocephalus***	▲	▲		▲	▲
	Rodentia	Muridae	**Muridae sp.**		●			
	Rodentia	Gliridae	***Graphiurus kelleni***					●
	Rodentia	Gliridae	***Graphiurus microtis***					●
	Rodentia	Muridae	***Pelomys fallax***	▲				
Reptiles	Testudines	Pelomedusidae	***Pelusios sinuatus***					●

Legend		
Marker	12S	●
	16S	▲
	Both	●▲
Seq. tech	Nanopore	
	Illumina	
	Both	

294

295 **Fig 2. Primary terrestrial vertebrate taxa detected.** Detections are depicted across the five Zambian
296 sampling sites eDNA primers sets (12S or 16S rRNA, or both) and sequencing technology (nanopore
297 and Illumina sequencing, or both).

298 We next compared all our taxa detections between the different sequencing technologies and
299 camera trapping (Fig 3.). Briefly, camera trapping-based detection was biased towards birds
300 and mammals with 10 and 9 species detected, respectively, and no detections for amphibians
301 or reptiles (S3 Table) while eDNA-based methods yielded 25 detections across all four primary
302 terrestrial vertebrates, out of which 14 were at the species level and 5 at the genus and 7 at
303 the family level, respectively. We observed little overlap between the taxa detected by camera
304 traps and eDNA; this overlap increased slightly with taxonomic ranks (Fig 3).

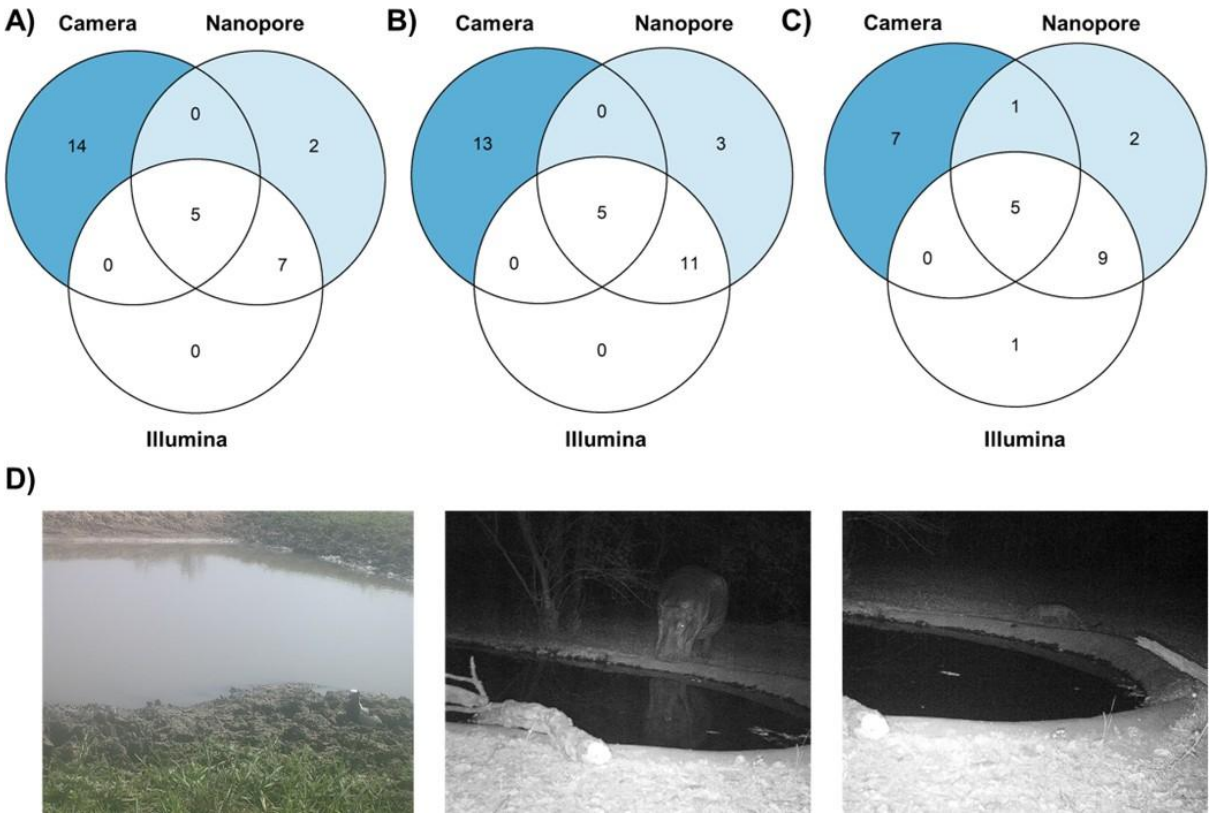

Fig 3. Shared taxa detections between camera trap-based biodiversity monitoring and freshwater-based eDNA-based monitoring using nanopore and Illumina sequencing. Taxonomic A) species, B) genus, and C) family detections. D) Examples of camera trap pictures: *left*: Blacksmith lapwing (*Vanellus armatus*) detected by all three approaches; *middle*: Hippopotamus (*Hippopotamus amphibius*) detected by all three approaches; and *right*: Small-spotted Genet (*Genetta genetta*) detected by camera trapping and by nanopore sequencing on the family level (*Viverridae sp.).*

We finally validated a full mobile laboratory workflow based on nanopore sequencing under field conditions (Methods). Over six consecutive sampling days at three sites of Mwendi Lagoon, we detected ten primary terrestrial vertebrate taxa using eDNA, and seven with camera traps, with four of the taxa detected by both methods (Fig 4A). Within the eDNA detections, nine taxa were recovered with the 12S rRNA primer, and four with the 16S rRNA primer, including three taxa amplified by both primers (Fig 4B). No local species was detected in the negative controls. Taxonomic richness varied across the three sites: Site A yielded the highest richness by eDNA approaches (n = 9), followed by sites B and C (n = 4 and n = 3, respectively); more taxa could be detected by eDNA than by camera trapping at A and C, and *vice versa* at B (Fig 4C).

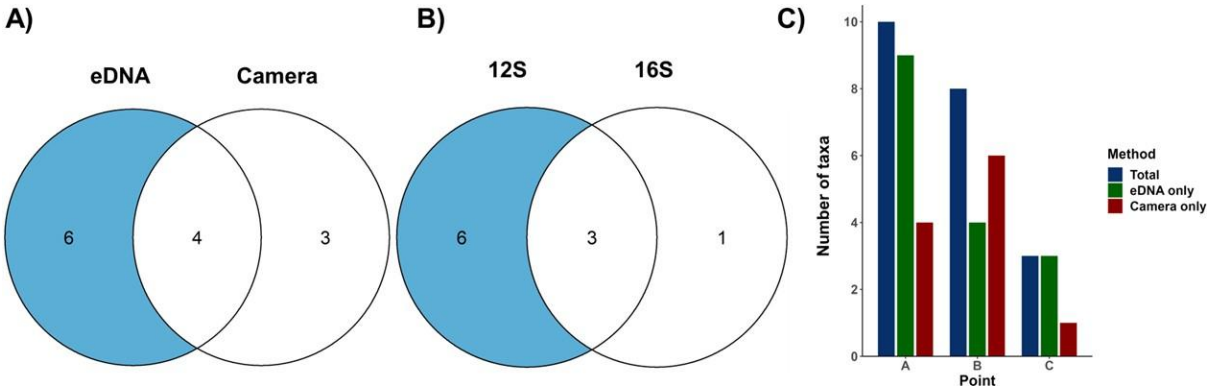

Fig 4. Number of primary terrestrial vertebrate taxa accumulated over six days detected. A) *in situ* eDNA and camera trapping; B) the two different primers used for *in situ* eDNA (12S and 16S rRNA); and C) by all approaches across the three sampling sites at the Mwendi Lagoon, Zambia.

We found that frequent visitors (e.g., *Loxodonta africana*, *Papio. cynocephalus*) and semi-aquatic taxa (e.g., *H. amphibius*, *Xenopus sp*.) were detected on multiple days and by more than one approach, whereas birds, bats, and rodents were typically recorded on a single day and by only one method. All antelopes—*Aepyceros. melampus, Tragelaphus. scriptus, Kobus. vardonii*, and *Kobus. ellipsiprymnus*—were detected exclusively by camera trapping and were not recovered from eDNA samples (Fig 5).

Class	Order	Species	Method	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Amphibians	Anura	*Hyperolius sp.*	eDNA					●	●
			Camera traps	NA					
	Anura	*Xenopus sp.*	eDNA					●	
			Camera traps	NA					
Birds	Galliformes	*Numida meleagris*	eDNA			●			
			Camera traps	NA					
	Pelecaniformes	*Threskiornis aethiopicus*	eDNA						●
			Camera traps	NA					
Mammals	Artiodactyla	*Hippopotamus amphibius*	eDNA	●▲	●▲	●▲	●▲	●▲	●▲
			Camera traps	NA					
	Artiodactyla	*Aepyceros melampus*	eDNA						
			Camera traps	NA					
	Primates	*Papio cynocephalus*	eDNA			●		●	
			Camera traps	NA					
	Chiroptera	*Epomophorus pusillus*	eDNA				●		
			Camera traps	NA					
	Rodentia	*Thallomys paedulcus*	eDNA						▲
			Camera traps	NA					
	Proboscidea	*Loxodonta africana*	eDNA	▲	▲	▲	▲		
			Camera traps	NA					
Amphibians	Anura	*Xenopus sp.*	eDNA	●		●	●	●	●
			Camera traps	NA					
Birds	Galliformes	*Numida meleagris*	eDNA						
			Camera traps	NA					
	Passeriformes	*Vidua macroura*	eDNA				●		
			Camera traps	NA					
Mammals	Artiodactyla	*Hippopotamus amphibius*	eDNA	●▲	●▲	●▲	●▲	▲	●▲
			Camera traps	NA					
	Artiodactyla	*Kobus vardonii*	eDNA						
			Camera traps	NA					
	Artiodactyla	*Tragelaphus scriptus*	eDNA						
			Camera traps	NA					
	Artiodactyla	*Kobus ellipsiprymnus*	eDNA						
			Camera traps	NA					
	Primates	*Papio cynocephalus*	eDNA	●▲	●▲		●▲	●▲	
			Camera traps	NA					
Amphibians	Anura	*Xenopus sp.*	eDNA	●	●	●	●	●	●
			Camera traps	NA					
Mammals	Artiodactyla	*Hippopotamus amphibius*	eDNA	●▲	●▲	●▲	●▲	●▲	●▲
			Camera traps	NA					
	Proboscidea	*Loxodonta africana*	eDNA	●▲	▲	▲			
			Camera traps	NA					

Legend		
Point	A	
	B	
	C	
Marker	12S	●
	16S	▲
	Both	●▲

Fig 5. Primary terrestrial vertebrate taxa detected by *in situ* eDNA and camera trapping. Detections are shown for three sampling point (A, B, C) at the Mwendi Lagoon, Zambia, across six consecutive sampling days (Methods). For eDNA detections, the primers 12S (●) and 16S (▲) rRNA are indicated.

Discussion

While eDNA approaches hold promise for providing a cost- and time-efficient alternative to conventional biodiversity monitoring approaches such as camera trapping, the *in situ* application of eDNA in biodiverse areas and countries has so far been hampered by the reliance of standard protocols on Illumina sequencing technology, which requires expensive machinery that is mostly only available at centralized sequencing facilities or research laboratories [4,5,21]. It is, however, important to conduct eDNA analyses on site—to obtain results without delay, empower local decision-makers, and to fulfill international treaties on

346 data sovereignty, material export, and benefit sharing when it comes to genetics-based
347 biodiversity monitoring [14,15].

348 Here, we established portable nanopore sequencing for eDNA analysis of freshwater samples
349 from Zambia for the detection of primary terrestrial vertebrate species and then compared
350 established Illumina and nanopore sequencing-based eDNA results with each other based on
351 the same DNA extracts and the same targeted marker gene regions. We show that the
352 improvements in nanopore sequencing accuracy through sequencing chemistry updates
353 (R10.4.1) and improved data basecalling algorithms (Dorado SUP) lead to mostly comparable
354 species detections between the two eDNA approaches, with nanopore sequencing even
355 detecting more local species than Illumina sequencing. Based on these results, we applied a
356 fully mobile approach based on the portable nanopore genomic technology and cost-effective
357 sample multiplexing for *in situ* biodiversity monitoring on site in the Luambe National Park in
358 Zambia, confirming the *in situ* applicability of our eDNA approach.

359 Due to its long sequencing reads, nanopore sequencing further holds promise for extending
360 future metabarcoding studies to longer marker gene regions, or entire mitochondrial genomes
361 [18,21,22,38]. For example, Bludau *et al.* showed that nanopore-sequenced amplicons of the
362 full-length 18S rRNA gene from soil protistan communities achieved greater taxonomic
363 classification accuracy down to the species level than short-read Illumina-sequenced
364 amplicons of the Variable Region 9 of the same gene, which only provided reliable
365 classification on the phylum level [18]. Similarly, Doorenspleet, *et al.* compared nanopore-
366 sequenced amplicons of a 2 kb region of fish mitochondrial DNA against the commonly used
367 MiFish primer pair targeting a ~170 bp region. They showed that while the longer amplicons
368 allowed for more species assignment in a controlled aquarium condition, the shorter amplicons
369 performed better in natural environmental settings. This is probably because eDNA is often
370 highly fragmented due to various environmental factors such as UV radiation, microbial
371 activity, and enzymatic degradation, resulting in higher persistence time for shorter fragments
372 [39-41]; such shorter DNA fragments are furthermore likely to amplify during PCR reactions

373 [42]. When using eDNA to monitor vertebrates, especially primary terrestrial vertebrates from
374 freshwater samples like presented in our study, the samples' DNA might therefore be too
375 fragmented to allow for efficient long-read gene sequencing, which remains to be assessed.
376 The suitability of amplicon length in metabarcoding studies might ultimately depend on
377 different factors, such as the targeted taxa, the eDNA source, and the amount of genetic
378 variability contained within the amplified marker. In highly diverse regions (e.g., the Luangwa
379 Valley) that host several sympatric closely related species, for example among antelopes and
380 felids, the use of longer amplicons could provide the taxonomic resolution needed to
381 discriminate among species.

382 Based on our database exploration, we found that, even for small marker gene regions (such
383 as the 12S and 16S rRNA regions used in this study), genomic databases remain incomplete,
384 particularly for megadiverse but understudied ecosystems [42-44]—such as Zambia's
385 subtropical savannahs ecosystems as assessed in this study. The accurate mapping of
386 marker gene OTUs to publicly available databases such as the entire NCBI nucleotide or the
387 MIDORI2 databases strongly depends on the regional taxonomic coverage and can lead to
388 unassigned sequences or false-positive assignments in the case of lacking references [45-
389 47]. We therefore emphasize that building or populating genetic databases with regional
390 genetic diversity is crucial for the successful application of eDNA based monitoring
391 approaches; until databases are more complete, any eDNA application must be interpreted
392 while taking the availability of genetic information into account.

393 We further compared our eDNA approaches with more conventional biodiversity monitoring
394 achieved through simultaneously applied camera trapping Both our eDNA approaches
395 showed little overlap with camera-based species detections. Several methodological and
396 ecological factors could explain the discrepancies. As discussed previously, database
397 completeness, primer choice, and amplicon resolution can influence which taxa are detected
398 by eDNA. In addition, the interaction between species and the eDNA source can determine
399 the likelihood of detection. For example, three recent studies have shown that waterborne

400 eDNA from African waterholes can successfully reveal mammalian and other vertebrate
401 communities [27-29]. However, we argue that these methods are likely to perform better for
402 certain species than others, depending on their ecology. Water-dwelling or drought-intolerant
403 species, such as many mammal or bird species, are more likely to leave detectable DNA
404 traces in such waterholes. eDNA persistence in waterholes has further shown to be relatively
405 short (Farell, 2022), which can contribute to variability in detection rates among species. Our
406 findings illustrate this ecological bias, with eDNA preferentially detecting species with frequent
407 or prolonged contact with water, such as hippos, elephants, and amphibians. Species that
408 interact only briefly with water bodies, such as antelopes and felids, may not shed sufficient
409 DNA to be consistently detected by eDNA, but could reliably be detected by camera trapping.
410 Similarly, drought-resistant species or those able to obtain water from food resources may
411 leave little or no trace in water samples. On the other hand, camera traps might miss species
412 that cannot be easily detected visually, such as, for example, reptiles, amphibians, and small
413 mammals such as rodents, bats, and pangolins. Overall, the limited overlap between eDNA
414 and camera trapping suggests their complementary application and highlights the value of
415 integrating additional eDNA sources such as air, soil, or blood-feeding insects [48-52].

416 In summary, we show that nanopore sequencing technology can be used to reliably carry out
417 eDNA-based biodiversity monitoring in remote locations, including in highly diverse LMICs
418 such as Zambia. The mobile laboratory and protocols we describe can be easily adapted for
419 processing other eDNA sources, or for other conservation purposes such as pathogen
420 surveillance. These efforts can pave the way towards democratization of genomic technology
421 in general, and eDNA applications specifically. However, further efforts are needed to (i) make
422 eDNA-based approaches more cost-effective so that capacity can be built for routine
423 applications in LMICs, particularly for conservation purposes where resources are often
424 limited, and to (ii) contribute to regional reference database developments to better reflect
425 global genetic biodiversity.

426

Supplementary Material

All supplementary material is currently available here. The code used for this study is available at: https://github.com/dmgr90/eDNA-Zambia-1

Data Accessibility Statement

All sequencing data is deposited in the NCBI Sequence Read Archive (SRA; accession number: PRJNA1195924).

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437 **References**

438 1. Butchart, S. H. M., Walpole, M., Collen, B., van Strien, A., Scharlemann, J. P. W., Almond,
439 R. E. A., Baillie, J. E. M., Bomhard, B., Brown, C., Bruno, J., Carpenter, K. E., Carr, G. M.,
440 Chanson, J., Chenery, A. M., Csirke, J., Davidson, N. C., Dentener, F., Foster, M., Galli, A.,
441 … Watson, R. 2010;. Global Biodiversity: Indicators of Recent Declines. Science, 328(5982),
442 1164–1168. doi: 10.1126/science.118751
443
444 2. Kindsvater, H. K., Dulvy, N. K., Horswill, C., Juan-Jordá, M.-J., Mangel, M., & Matthiopoulos,
445 J. 2018;. Overcoming the Data Crisis in Biodiversity Conservation. Trends in Ecology &
446 Evolution, 33(9), 676–688. doi: 10.1016/j.tree.2018.06.00
447
448 3. Vié, J.-C., Hilton-Taylor, C., & Stuart, S. N. 2009;. Wildlife in a changing world: An analysis
449 of the 2008 IUCN red list of threatened species. IUCN ; Lynx Edicions
450
451 4. Lewis, D. 2019;. Rare bird's detection highlights promise of 'environmental DNA'. Nature,
452 575(7783), 423–424. doi: 10.1038/d41586-019-03522-
453
454 5. Matthias, L., Allison, M. J., Maslovat, C. Y., Hobbs, J., & Helbing, C. C. 2021;. Improving
455 ecological surveys for the detection of cryptic, fossorial snakes using eDNA on and under
456 artificial cover objects. Ecological Indicators, 131, 108187. doi: 10.1016/j.ecolind.2021.10818
457
458 6. Mizumoto, H., Mitsuzuka, T., & Araki, H. 2020;. An Environmental DNA Survey on
459 Distribution of an Endangered Salmonid Species, Parahucho perryi, in Hokkaido, Japan.
460 Frontiers in Ecology and Evolution, 8, 569425. doi: 10.3389/fevo.2020.56942
461
462 7. Itakura, H., Wakiya, R., Yamamoto, S., Kaifu, K., Sato, T., & Minamoto, T. 2019;.
463 Environmental DNA analysis reveals the spatial distribution, abundance, and biomass of

Japanese eels at the river-basin scale. Aquatic Conservation: Marine and Freshwater Ecosystems, 29(3), 361–373. doi: 10.1002/aqc.3058

8. Nardi, C. F., Fernández, D. A., Vanella, F. A., & Chalde, T. 2019;. The expansion of exotic Chinook salmon (Oncorhynchus tshawytscha) in the extreme south of Patagonia: An environmental DNA approach. Biological Invasions, 21(4), 1415–1425. doi: 10.1007/s10530-018-1908-8

9. Sales, N. G., Wangensteen, O. S., Carvalho, D. C., Deiner, K., Præbel, K., Coscia, I., McDevitt, A. D., & Mariani, S. 2021;. Space-time dynamics in monitoring neotropical fish communities using eDNA metabarcoding. Science of The Total Environment, 754, 142096. doi: 10.1016/j.scitotenv.2020.142096

10. Fisher, B., & Christopher, T. 2007;. Poverty and biodiversity: Measuring the overlap of human poverty and the biodiversity hotspots. Ecological Economics, 62(1), 93–101. doi: 10.1016/j.ecolecon.2006.05.020

11. Urban, L., Perlas, A., Francino, O., Martí-Carreras, J., Muga, B. A., Mwangi, J. W., Boykin Okalebo, L., Stanton, J. L., Black, A., Waipara, N., Fontsere, C., Eccles, D., Urel, H., Reska, T., Morales, H. E., Palmada-Flores, M., Marques-Bonet, T., Watsa, M., Libke, Z., ... Van Oosterhout, C. 2023;. Real-time genomics for One Health. Molecular Systems Biology, e11686. doi: 10.15252/msb.20231168

12. Watsa, M., Erkenswick, G. A., Pomerantz, A., & Prost, S. 2020;. Portable sequencing as a teaching tool in conservation and biodiversity research. PLOS Biology, 18(4), e3000667. doi: 10.1371/journal.pbio.3000667

13. Convention on International Trade in Endangered Species of Wild Fauna and Flora | CITES. (n.d.). Retrieved December 6, 2024, from https://cites.org/eng/disc/text.php

14. The Nagoya Protocol on Access and Benefit-sharing. (n.d.). Retrieved December 6, 2024, from https://www.cbd.int/ab

15. CARE Principles—Global Indigenous Data Alliance. (n.d.). Retrieved December 6, 2024, from https://www.gida-global.org/care

16. Urban, L., Miller, A. K., Eason, D., Vercoe, D., Shaffer, M., Wilkinson, S. P., Jeunen, G.-J., Gemmell, N. J., & Digby, A. 2022;. Non-invasive real-time genomic monitoring of the critically endangered kākāpō [Preprint]. Genomics. doi: 10.1101/2022.11.14.516431

17. Baloğlu, B., Chen, Z., Elbrecht, V., Braukmann, T., MacDonald, S., & Steinke, D. 2021;. A workflow for accurate metabarcoding using nanopore MinION sequencing. Methods in Ecology and Evolution, 12(5), 794–804. doi: 10.1111/2041-210X.13561

18. Bludau, D., Sieber, G., Shah, M., Deep, A., Boenigk, J., & Beisser, D. 2025;. Breaking the Standard: Can Oxford Nanopore Technologies Sequencing Compete With Illumina in Protistan Amplicon Studies? Environmental DNA, 7(2), e70084. doi: 10.1002/edn3.70084

19. Van Der Reis, A. L., Beckley, L. E., Olivar, M. P., & Jeffs, A. G. 2023;. Nanopore short-read sequencing: A quick, cost-effective and accurate method for DNA metabarcoding. Environmental DNA, 5(2), 282–296. doi: 10.1002/edn3.374

20. Egeter, B., Veríssimo, J., Lopes-Lima, M., Chaves, C., Pinto, J., Riccardi, N., Beja, P., & Fonseca, N. A. 2021;. Metabarcoding with MinION: Speeding up the detection of invasive

518 aquatic species using environmental DNA and nanopore sequencing. ARPHA Conference
519 Abstracts, 4, e65036. doi: 10.3897/aca.4.e6503
520
521 21. Bista, I., & Lino, A. 2025. Long-read sequencing for biodiversity analyses – a
522 comprehensive guide. EcoEvoRxiv (preprint), published August 26, 2025.
523
524 22. Doorenspleet, K., Jansen, L., Oosterbroek, S., Kamermans, P., Bos, O., Wurz, E., Murk,
525 A., & Nijland, R. 2025;. The Long and the Short of It: Nanopore-Based eDNA Metabarcoding
526 of Marine Vertebrates Works; Sensitivity and Species-Level Assignment Depend on Amplicon
527 Lengths. Molecular Ecology Resources, 25(4), e14079. doi: 10.1111/1755-0998.1407
528
529 23. Government of the Republic of Zambia. 2015;. Zambia's second national biodiversity
530 strategy and action plan (NBSAP-2) 2015–2025. Ministry of Lands, Natural Resources and
531 Environmental Protection.
532
533 24. Critical Ecosystem Partnership Fund (CEPF). Final Project Completion Report: Project
534 Number SG68954. 2
535 017. Available from: https://www.cepf.net/sites/default/files/final-report-sg68954.pdf
536
537 25. Center for Large Landscape Conservation (CLLC). 2020;. Zambia FOCUS-BRI Report.
538 Retrieved from https://largelandscapes.org/wp-content/uploads/Zambia-FOCUS-BRI.pdf
539
540 26. Thrash, I., Theron, G. K., & du Bothma, J. P. 1995; Dry season herbivore densities around
541 drinking troughs in the Kruger National Park. *Journal of Arid Environments, 29*(2), 213–219.
542 doi: 10.1016/S0140-1963(05)80091-6
543

27. Farrell, M. J., Govender, D., Hajibabaei, M., Van Der Bank, M., & Davies, T. J. 2022; Environmental DNA as a management tool for tracking artificial waterhole use in savanna ecosystems. *Biological Conservation, 274,* 109712. doi: 10.1016/j.biocon.2022.109712

28. Seeber, P. A., McEwen, G. K., Löber, U., Förster, D. W., East, M. L., Melzheimer, J., & Greenwood, A. D. 2019; Terrestrial mammal surveillance using hybridization capture of environmental DNA from African waterholes. *Molecular Ecology Resources, 19,* 1486–1496. doi: 10.1111/1755-0998.13069

29. Schenekar, T., Baxter, J., Phukuntsi, M. A., Sedlmayr, I., Weckworth, B., & Mwale, M. 2024; Optimizing waterborne eDNA capture from waterholes in savanna systems under remote field conditions. *Molecular Ecology Resources, 24*(4), e13942. doi: 10.1111/1755-0998.13942

30. Riaz, T., Shehzad, W., Viari, A., Pompanon, F., Taberlet, P., & Coissac, E. 2011;. ecoPrimers: Inference of new DNA barcode markers from whole genome sequence analysis. Nucleic Acids Research, 39(21), e145–e145. doi: 10.1093/nar/gkr732

31. Taylor, P. G. 1996; Reproducibility of ancient DNA sequences from extinct Pleistocene fauna. *Molecular Biology and Evolution*. 13(1): 283–285. doi: 10.1093/oxfordjournals.molbev

32. Srivathsan, A., Feng, V., Suárez, D., Emerson, B., & Meier, R. 2023;. Rapid species discovery and identification with real-time barcoding facilitated by ONTbarcoder 2.0 and Oxford Nanopore R10.4 [Preprint]. Ecology. doi: 10.1101/2023.06.26.546538

33. Martin, M. 2011; Cutadapt Removes Adapter Sequences from High-Throughput Sequencing Reads. EMBnet Journal, 17, 10-12

572 34. Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. 2016;. VSEARCH: A versatile
573 open source tool for metagenomics. PeerJ, 4, e2584. doi: 10.7717/peerj.258
574
575 35. Leray, M., Knowlton, N., & Machida, R. J. 2022;. MIDORI2: A collection of quality
576 controlled, preformatted, and regularly updated reference databases for taxonomic
577 assignment of eukaryotic mitochondrial sequences. *Ecology and Evolution*, 12(4), e0303. doi:
578 10.1002/edn3.303
579
580 36. Calvignac-Spencer, S., Merkel, K., Kutzner, N., Kühl, H., Boesch, C., Kappeler, P. M.,
581 Metzger, S., Schubert, G., & Leendertz, F. H. 2013; Carrion fly-derived DNA as a tool for
582 comprehensive and cost-effective assessment of mammalian biodiversity. *Molecular Ecology*.
583 22(4): 915–924. doi: 10.1111/mec.12183
584
585 37. Vestheim, H., & Jarman, S. N. 2008; Blocking primers to enhance PCR amplification of
586 rare sequences in mixed samples – a case study on prey DNA in Antarctic krill stomachs.
587 *Frontiers in Zoology*. 5: 12. doi: 10.1186/1742-9994-5-12
588
589
590 38. Urban, L., Holzer, A., Baronas, J. J., Hall, M. B., Braeuninger-Weimer, P., Scherm, M. J.,
591 Kunz, D. J., Perera, S. N., Martin-Herranz, D. E., Tipper, E. T., Salter, S. J., & Stammnitz, M.
592 R. 2021;. Freshwater monitoring by nanopore sequencing. eLife, 10, e61504. doi:
593 10.7554/eLife.61504
594
595 39. Bista, I., Carvalho, G. R., Tang, M., Walsh, K., Zhou, X., Hajibabaei, M., Shokralla, S.,
596 Seymour, M., Bradley, D., Liu, S., Christmas, M., & Creer, S. 2018;. Performance of amplicon
597 and shotgun sequencing for accurate biomass estimation in invertebrate community samples.
598 Molecular Ecology Resources, 18(5), 1020–1034. doi: 10.1111/1755-0998.1288
599

600 40. Deagle, B. E., Jarman, S. N., Coissac, E., Pompanon, F., & Taberlet, P. 2014;. DNA
601 metabarcoding and the cytochrome c oxidase subunit I marker: Not a perfect match. Biology
602 Letters, 10(9), 20140562. doi: 10.1098/rsbl.2014.056
603
604 41. Jo, T., Murakami, H., Masuda, R., Sakata, M. K., Yamamoto, S., & Minamoto, T. 2017;.
605 Rapid degradation of longer DNA fragments enables the improved estimation of distribution
606 and biomass using environmental DNA. Molecular Ecology Resources, 17(6). doi:
607 10.1111/1755-0998.12685
608
609 42 Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F.,
610 Creer, S., Bista, I., Lodge, D. M., Vere, N., Pfrender, M. E., & Bernatchez, L. 2017;.
611 Environmental DNA metabarcoding: Transforming how we survey animal and plant
612 communities. Molecular Ecology, 26(21), 5872–5895. doi: 10.1111/mec.14350
613
614 43. Li, F., Zhang, Y., Altermatt, F., Zhang, X., Cai, Y., & Yang, Z. 2022;. Gap analysis for DNA-
615 based biomonitoring of aquatic ecosystems in China. Ecological Indicators, 137, 108732. doi:
616 10.1016/j.ecolind.2022.10873
617
618 44. Marques, V., Milhau, T., Albouy, C., Dejean, T., Manel, S., Mouillot, D., & Juhel, J. 2021;.
619 GAPeDNA: Assessing and mapping global species gaps in genetic databases for eDNA
620 metabarcoding. Diversity and Distributions, 27(10), 1880–1892. doi: 10.1111/ddi.13142
621
622 45. Supple, M. A., & Shapiro, B. 2018;. Conservation of biodiversity in the genomics era.
623 Genome Biology, 19(1), 131. doi: 10.1186/s13059-018-1520-3
624

46. Keck, F., Couton, M., & Altermatt, F. 2023;. Navigating the seven challenges of taxonomic reference databases in metabarcoding analyses. Molecular Ecology Resources, 23(4), 742–755. doi: 10.1111/1755-0998.13746

47. Mugnai, F., Costantini, F., Chenuil, A., Leduc, M., Gutiérrez Ortega, J. M., & Meglécz, E. 2023;. Be positive: Customized reference databases and new, local barcodes balance false taxonomic assignments in metabarcoding studies. PeerJ, 11, e14616. doi: 10.7717/peerj.1461

48 Polling, M., Buij, R., Laros, I., & de Groot, G. A. 2024;. Continuous daily sampling of airborne eDNA detects all vertebrate species identified by camera traps. Environmental DNA, First published: 22 August 2024. doi: 10.1002/edn3.591

49. Lynggaard, C., Frøslev, T. G., Johnson, M. S., Olsen, M. T., & Bohmann, K. 2024;. Airborne environmental DNA captures terrestrial vertebrate diversity in nature. Molecular Ecology Resources, 24(1), e13840. doi: 10.1111/1755-0998.13840

50. Urban, L., Miller, A. K., Eason, D., Vercoe, D., Shaffer, M., Wilkinson, S. P., Jeunen, G.-J., Gemmell, N. J., & Digby, A. 2023;. Non-invasive real-time genomic monitoring of the critically endangered kākāpō. eLife, 12, RP84553. doi: 10.7554/eLife.84553.2

51. Vieira, C. J. S. P., Gyawali, N., Onn, M. B., et al. 2024;. Mosquito bloodmeals can be used to determine vertebrate diversity, host preference, and pathogen exposure in humans and wildlife. Scientific Reports, 14, 23203. doi: 10.1038/s41598-024-73820-y

52. Reeves, L. E., Gillett-Kaufman, J. L., Kawahara, A. Y., & Kaufman, P. E. 2018;. Barcoding blood meals: New vertebrate-specific primer sets for assigning taxonomic identities to host DNA from mosquito blood meals. PLoS Neglected Tropical Diseases, 12(8), e0006767. doi: 10.1371/journal.pntd.0006767

653