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Environmental DNA metabarcoding reveals the presence of invasive and cryptic species in the Mweru-Luapula fishery

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ABSTRACT

Environmental DNA (eDNA) has in recent years emerged as a major approach to complement traditional sampling methods across aquatic freshwater systems. Despite this increasing application of eDNA metabarcoding approach, a number of developing countries have not yet fully incorporated the tool in the management and monitoring of aquatic biodiversity. This study aimed at analysing eDNA water samples collected for the first time across 18 sampling sites of the Mweru-Luapula (ML) fishery to determine the presence and distribution of invasive and native freshwater fishes. The study further applied Simpson diversity indices (SDIs) to investigate the diversity of species between invaded and non-invaded systems. Environmental DNA analysis revealed the presence of invasive *Parachanna* species in three of the four strata of the fishery, compared to only two strata previously known to have been invaded when assessed by traditional methods. In addition, five rare species (*Marcusenius senegalensis, Trachurus japonicus, Labeo nasus, Campylomormyrus compressirostris and Synodontis schoutedeni*) were also initially detected using eDNA. Low SDI values were recorded in invaded individual sampling sites. The coefficient association between read counts and species frequencies ($r = 0.31$; p-value $= 0.239$) and diversity indices ($r = 0.1$; p-value $= 0.717$) did not have any significant impact. This study has provided a platform for further investigations on the presence and impact of invasive species in other fishery areas across the country, using eDNA water samples collected at different water depths to update the species inventories. The revelation of unexpected species for the first time in the fishery and detection of invasive *Parachanna* species in multiple sites has demonstrated the need for introducing eDNA metabarcoding alongside conventional methods to monitor alien invasive species and thus effectively manage and conserve the threatened aquatic biodiversity of the freshwater ML fishery of Zambia.

Introduction

Environmental DNA (eDNA) has in the recent past been regarded among the most reliable and cost-effective biodiversity monitoring tools in developed countries [1–3]. However, this is not yet the case in most third-world countries, including Zambia, dependent

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on traditional conventional methods. Common traditional and fishery independent fish stock assessment in Zambia include gill net surveys (GNSs), catch assessment surveys (CASs), frame surveys, market statistics and limnological studies [4].

Gill net surveys (GNSs) are traditional sampling techniques employed in the ML fishery for over five decades. These surveys provide data used for the evaluation of fish populations, thereby enhancing resource management in freshwater ecosystems [5]. A gill net fleet comprises nets with mesh sizes of 8, 12, 25, 32, 37, 50, 63, 76, 89, 102, 114, 127, 140, 152, 165, 192, 204, 216, 228, 240 and 253 mm. The fleet is set as either top set, middle set or bottom set – with a hanging ratio ranging between 0.5 and 0.7 [4].

Catch assessment survey (CAS) is a common and popular assessment tool used for fish stock management. This assessment is conducted through monitoring and data collection on fish species status in a water body of interest [6]. Catch assessment surveys can be conducted at selected sites following approved standard operating procedures. The CAS data is analysed in Excel using Bazigos [7] formula based on catch per unit of effort, activity rate, average gears/crew per boat and weight of the individual fish.

Traditional surveys are costly, laborious and cannot readily capture data on alien invasive and cryptic species [4]. To conduct both GNS and CAS, a team of personnel is required and the processes are also time consuming, as a fleet of connected gill nets is left overnight or for a period of not less than 8 h during daylight before hauling [4]. If wind is experienced in the course of hauling, the nets will be hauled in the direction of the wind or current. Information obtained from these conventional surveys is also inadequate for evaluating the effects of invasive species on the biodiversity and surveying all fishery areas of the ML fishery. Furthermore, the traditional methods are inefficient and prone to non-detection error [3].

Limitations and knowledge gap from conventional methods could sufficiently be addressed through the use of environmental DNA (eDNA) metabarcoding. In this study, eDNA metabarcoding refers to the molecular analysis of DNA obtained from environmental water samples to deduce the presence of fish species [1,8]. It is an appropriate lower-cost bioassessment tool that can assist in addressing the effects of climate change and escalating anthropogenic impact on natural ecosystems [9]. The new approach can be enhanced by focussed species-specific surveys [10]. The application of eDNA abundance data is expected to correlate consistently with abundance estimates from conventional methods or established surveys [11]. Furthermore, eDNA supplements some limitations arising from applying conventional or traditional methods in stock assessment [3]. The emergence of eDNA metabarcoding is slowly addressing some shortfalls from conventional methods [11]. We are now, through the use of eDNA, able to witness the alarming levels at which non-native species are invading vast ecosystems [12]. Early detection of alien species in an environment before a population is established can assist managers in mitigating effects and damage to invaded environments. However, this requires sensitive surveying tools such as eDNA, which can even track cryptic species [3,12,13].

Through extensive trials and optimisation, eDNA metabarcoding has become a reliable tool for assessing aquatic and terrestrial biodiversity [14]. Unlike visual and capture-based methods, eDNA does not involve any form of selection but utilises the availability of genetic material in the sampled media [15]. The approach can also assess the state of biodiversity and thus distinguish specific native and invasive fish species in a particular area of interest [16]. One of the major breakthroughs in characterising eDNA from water samples is the ability to deduce the presence, distribution and diversity of organisms without physically handling or observing them [17]. The eDNA metabarcoding tool provides qualitative evidence of species' occupancy and can be used as a quantitative measure or a relative indication of abundance [18]. Additionally, when eDNA metabarcoding is used along with a fine-scale sampling design, it can give an indicative status of the entire biodiversity [19]. Apart from its efficacy in detecting taxa, eDNA analysis can similarly be applied to exploit innovative perceptions in ecology [15].

In freshwater streams, eDNA metabarcoding has proven more accurate and sensitive than traditional electrofishing in fish monitoring surveys [2]. This underscores the essence and huge potential of applying eDNA metabarcoding in biomonitoring vast freshwater and marine ecosystems [20]. The tool surpasses all other monitoring methods because of its ability to assess the diversity of functional traits and its power to detect species [15]. This biomonitoring instrument, alongside next-generation sequencing methods, can enable extensive parallel sequencing of mixed samples. By using marker genes (such as the rRNA genes) with conserved sequences across diverse taxa for primer design yet variable enough to detect differences, one can screen a broad range of potential species across families, orders and phyla present in a system [21]. Environmental DNA can also be employed to monitor alien/invasive and endangered freshwater fish species [3], leading to the development of appropriate strategies to obtain adequate data on aquatic organisms from water samples [16]. The method can detect closely interrelated species in diverse aquatic communities within evolutionary radiations [22].

Recently, the probability of species detection from eDNA samples has proved to be more accurate and reliable compared to traditional or conventional methods. For instance, Sigsgaard et al. [3] detected the presence of European weather loach *Misgurnus fossilis* at numerous sites that traditional methods failed to trace in the past two decades of routine monitoring surveys. Gillet et al. [13] also demonstrated a higher sensitivity in species identification by detecting 30 more species, which could no longer be detected by conventional methods. The species were originally known to be present at the Nam Theun 2 sampling area, before the construction of the Nakai dam in Khammouan Province, Vietnam. GNS had never detected these species in the previous three years. In another study by Hänfling et al. [11], over 75 % of species in the English Lake District were detected using eDNA metabarcoding compared to less than 30 % detection recorded previously from GNS. Yamamoto et al. [23] spent six hours to collect water samples, and identified 128 fish species with eDNA metabarcoding, compared to 40 fish species which were captured by submerged visual censuses carried out over more than 14 years. In addition, environmental managers can map zones for species distribution by using eDNA assays, without the use of reports and monitoring from fishers and community members [24].

Indigenous fauna and flora have been threatened by four centuries of alien species invasion and habitat alterations [25]. This occurrence can ably be understood by a multiple approach to studying the vast affected species, ecosystems and genetic diversity. The study of genetic diversity and structure of species enables researchers to identify genes that determines the path of evolution as ascertained by Tao et al. [26] whose conclusions laid a platform for future exploration of disease, molecular breeding, significant traits

of economic importance and preservation of genetic resource among other issues. Lande [27] had earlier stressed how critical it is to maintain genetic diversity for the purpose of conserving threatened species and facilitate the recovery of species from unpredictable environmental and demographic changes. This agrees with what Osho et al. [28] revealed in affirming the range of nucleotide diversity among populations and intra-populations variation of *P. obscura* from the studied five rivers- Anambra, Ibbi, Imo, Katsina- Ala and Ogun of Nigeria. This information on genetic diversity can be gained by inferring haplotypes from sequences of field samples collected from diverse populations [29].

Despite the major strides made in applying sensitive eDNA metabarcoding in biomonitoring, the tool has been mainly confined to temperate ecosystems [22]. There is a need for a robust approach to adopt this innovation, even in tropical freshwater ecosystems found in many developing countries. This study aimed at demonstrating the essence and benefit of incorporating eDNA alongside traditional assessment methods to monitor and manage the vast freshwater aquatic resources of the ML fishery. This was done by analysing eDNA water samples collected to determine the presence and distribution of invasive *Parachanna* species, *Oreochromis* species, and other indigenous of the freshwater fishery. Furthermore, the study used Simpson diversity indices, DNA barcodes and phylogenetic analyses to investigate the diversity and relationship of species between impacted or invaded and non-impacted systems.

Materials and methods

Study area

The study was conducted in the ML fishery of Zambia, located at 8° 28′–9° 31′ S, and 28° 20′–29° 20′ E (Fig. 1) [30]. Ninety eDNA filtrates or water samples were obtained from 18 sampling sites, five replicates per site, over a period of 21 days in November 2020. The sampling sites were distributed throughout the four strata of the fishery and are designated Department of Fisheries (DoF) established monitoring points based on significant fish abundance and diversity. In accordance with the principles of Bazigos [7], ML fishery was divided into four different strata, a division that still stands to-date. These strata are geographical units and form the basis of statistical data collection, reflecting the differences in fish species composition and the type of fishery. Stratification of sampling sites was done to obtain representative samples from the officially designated and stratified areas of the ML fishery of Zambia. The 18

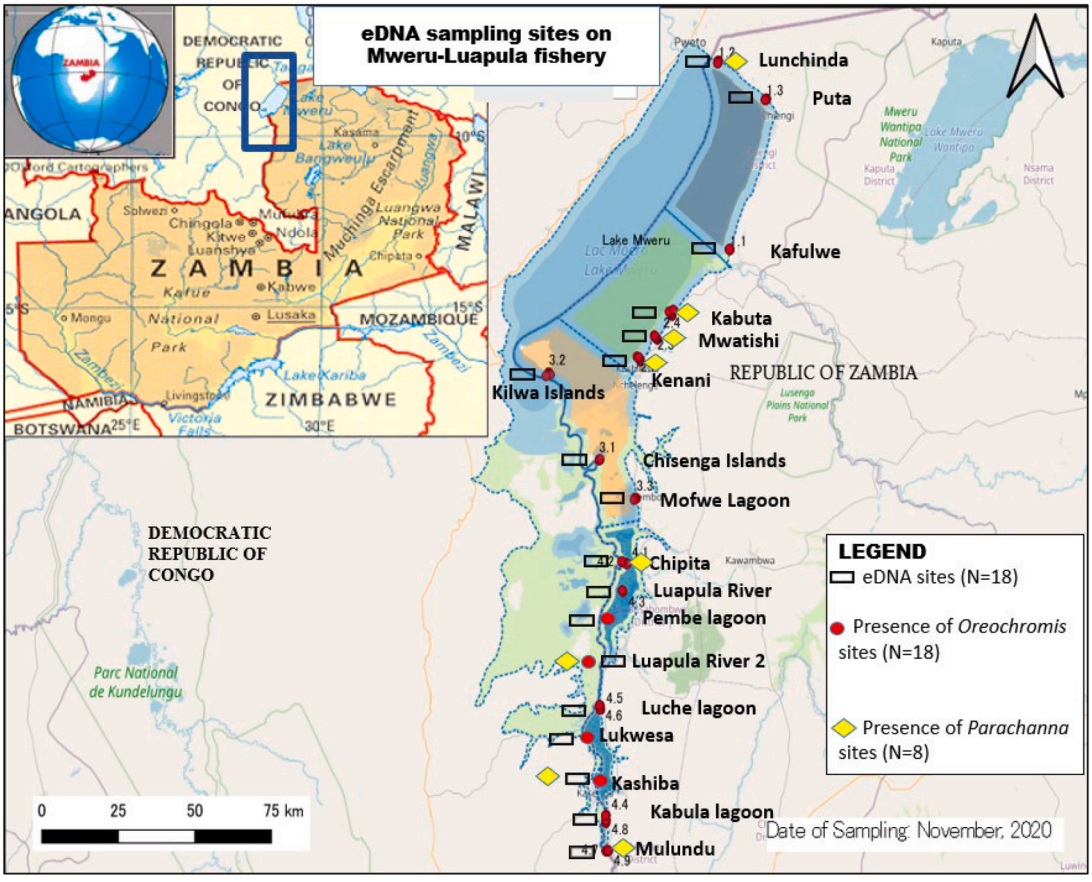

Fig. 1. The Mweru-Luapula fishery with the location of 18 eDNA sampling sites (designated by black rectangles). The insert shows the map of Zambia, depicting the location (purple rectangle) of the fishery.

sampling sites were Lunchinda-Lupiya, Kafulwe, Puta-Abinala (Stratum 1), Kenani, Mwatishi, Kabuta (Stratum 2), Chisenga Islands-Muku lagoon, Kilwa Islands, Mofwe Lagoon (Stratum 3), Filumba Lagoon-Chipita, Kabula Lagoon-Kashiba, Kashiba (LR), Luapula River (LR I), Luapula River (LR II),

eDNA samples collection

Water samples were collected from surface waters between 0 and 30 cm water depth, using 1L sterilized plastic sampling bottles [11] for the 18 targeted sites of the ML fishery. The fishery was selected for study as one of the three fishery areas in Zambia, shared with a neighbouring country, the Democratic Republic of Congo (DRC). The fishery is prone to invasion of alien invasive species from the Congo River Basin. Five replications from each sampling site were obtained at the intervals of 0, 200, 400, 600 and 800 m from the fishery shoreline. The 18 sampling sites were selected for being designated or representative sampling sites adopted by Department of Fisheries under the Ministry of Fisheries and Livestock for routine monitoring and management of the ML fishery using conventional methods. Distances between samples were guided by an on-board Garmin Etrex 10 Geographic positioning system (GPS) [11,31]. A hand-operated pump with a vacuum gauge fitted to a Sterlitech 47 mm glass microanalysis holder (glass frit support, 300 ml KG47) was used to filter water samples using a 47 mm diameter cellulose nitrate filter (pore size, 0.45 μm) directly on the research boat, within 24 h after collection [11]. A litre of distilled water in a sterilized plastic sampling bottle was used as a cooler blank or control, taken between sampling sites. This was done to minimize cross-contamination, including bleaching of all filtration equipment after every filtration run [31]. Filters were then carefully folded inwards three times and inserted into 1.8 μl serum vials. This was performed using forceps cleaned using 30 % bleach and rinsed with distilled water. The vials were filled with 95 % ethanol and sealed before being transported for further analysis [22,32]. The samples were stored at −20 °C in microtube storage boxes before DNA extraction [13].

eDNA extraction and amplification by PCR

Total genomic DNA was extracted from paper filters stored in 95 % ethanol using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, German) according to the manufacturer's protocols. The quality and quantity of DNA extracted were assessed by both a Nanodrop 1000 spectrophotometer (Thermo Scientific) and the Qubit® 2.0 Fluorometer using the dsDNA HS assay (Invitrogen). The mitochondrial 12S gene (252 bp) were PCR amplified in 15 μl reaction volumes using the Promega Green Master Mix (Promega) following the manufacturer's protocol The reaction contained 1 - 2 μl template DNA, 0.9 μl mM $MgCl_2$ (Thermo Fisher Scientific), 1 x PCR buffer, 0.2 mM dNTPs (Anatech Instruments) and 0.1 μl Super-Therm *Taq* polymerase (Separation Scientific), 1.5 μl of each fish primer (5 μM), MiFish-U-F (5′ GTCGGTAAAACTCGTGCCAGC 3′) and MiFish-U-R (5′ CATAGTGGGGTATCTAATCCCAGTTTG ‘3) [33] (Inqaba Biotechnical Industries). The total volume was made up of 5.8 – 6.8 μl of ddH_2O and 0.5 μl of a 0.2 μg/μl of bovine serum albumin (Inqaba Biotechnical Industries) [33]. The PCR programme consisted of initial denaturation for 2 min at 94 °C, 35 cycles of 30 s at 94 °C, primer annealing for 20 s at 51 °C–56 °C and 15 s of elongation at 72 °C, and a final elongation step of 5 min at 72 °C. All the PCR runs had negative controls to cross-check for possible contamination. PCR products (4 μl aliquots) were tested for amplification success using agarose gel electrophoresis with 1 % w/v agarose gels stained using GelRed (Anatech Instruments). Each gel contained a 100 bp size standard (Inqaba Biotechnical Industries) to assess amplicon size and relative concentration.

The PCRs were repeated three to five times for each site's representative sample and then pooled to limit bias. Two sample pools had to be omitted due to being below the recommended sample concentration thresholds. Sixteen of the 18 samples representing 16 sampling sites were pooled, cleaned or purified using Agencourt AMPure XP beads (Beckman Coulter) following the manufacturer's operation manual. The 16 pooled samples were shipped (cooled) to the Central Analytical Facility of Stellenbosch University for library construction and sequencing.

DNA quality control and data analysis

Library construction and sequencing quality control of 16 products were done using the Ion Torrent Platform at the Central Analytical Facility - DNA Sequencing Unit, Stellenbosch University. The libraries were sequenced on a single 530 chip to yield ~ 600 000 reads per sample.

Double stranded DNA (dsDNA) from the 12S fragments were assessed for fragment size distribution on the LabChip GX Touch 24 (PerkinElmer) using the X-mark chip and HT DNA NGS 3K reagent kit (PerkinElmer) according to the manufacturer's protocol CLS145098. PCR fragments were purified with 1.8x volume Agencourt™ AMPure™ XP reagent (Beckman Coulter) and eluted in 25μl water. The purified fragments were quantified on the Qubit 4.0 Fluorometer using the Qubit 1x dsDNA HS assay kit (ThermoFisher Scientific) according to the protocol MAN0017455. The quality and quantity of each PCR fragment pool were considered sufficient to proceed with library construction.

Library preparation and sequencing run

Library preparation was performed using 50 ng pooled amplicons for each sample using the Ion Plus Fragment Library Kit according to the protocol MAN0006846. Each purified, pooled PCR product was end repaired at room temperature for 20 min. The end repaired products were purified with 1.8x volume Agencourt™ AMPure™ XP (Beckman Coulter) reagent. The purified, end-repaired product was ligated to IonCode™ Barcode Adapters (ThermoFisher Scientific). The adapter-ligated, barcoded libraries were purified with 1.4x volume Agencourt™ AMPure™ XP reagent (Beckman Coulter). The purified adapter-ligated libraries were amplified on the

SimpliAmp™ thermal cycler (ThermoFisher Scientific) with an initial DNA dissociation step at 95 °C for 5 min and 5 amplification cycles consisting of template denaturation, primer annealing and product extension at 95 °C for 15 s, 58 °C for 15 s and 70 °C for 1 min, respectively. The amplified libraries were purified with 1.4x volume Agencourt™ AMPure™ XP (Beckman Coulter) reagent and quantified using the Library TaqMan™ Quantitation Kit according to the manufacturer's protocol MAN0015802. The qPCR amplification was performed using the StepOnePlus™ Real-time PCR system (ThermoFisher Scientific). Fragment size distribution for each library was determined on the LabChip GX Touch 24 (PerkinElmer) using the X-mark chip and HT DNA NGS 3K reagent kit (PerkinElmer) according to the manufacturer's protocol CLS145098.

Template preparation, enrichment and sequencing

Libraries were diluted to a target concentration of 60pM. The diluted, barcoded libraries were combined in equimolar amounts for template preparation using the Ion 510™ & Ion 520™ & Ion 530™ Chef Kit (ThermoFisher Scientific). Twenty-five microliters of diluted, pooled library were loaded onto the Ion Chef liquid handler (ThermoFisher Scientific) for template preparation and enrichment according to the protocol MAN0016854 REVF.0. Enriched ion sphere particles were loaded onto an Ion 530™ Chip (ThermoFisher Scientific). Massively parallel sequencing for all 16 PCR pools ($n = 16$) was performed on the Ion GeneStudio™ S5 Prime System (next generation sequencing platform - ThermoFisher Scientific) using sequencing solutions and reagents according to the protocol MAN0016854.

Species identification

Raw paired-end reads imported into Geneious Prime 2023.2.1 software were quality trimmed using the BBDUK plugin with the following parameters: *Minimum Quality (Q) score: 30, k-mer length: 27, Minimum overlap: 24, Minimum sequence length: 100 bp*. Trimmed reads were error corrected and normalized using BBNorm with sensitivity set to 'conservatively' and a k-mer depth of 40. Trimmed reads were merged using the BBMerge tool in Geneious with the merge rate set to 'High'. Merged reads of expected length (150–220 bp) were extracted into a new folder for subsequent analysis. To create an Operational Taxonomic Unit (OTU), denovo assembly with customized high stringent settings (*Sensitivity: Custom Sensitivity, Allow Gaps Maximum Per Read: 1 %, Minimum Overlap: 100, Ignore repeated words more than: 200 times, and Minimum overlap identity: 98 %*) was performed on length filtered reads. The assembled reads (consensus sequences) and unassembled reads (unused reads) generated during denovo assembly were used in the subsequent analysis. Prior to Basic Local Alignment Search Tool (BLAST) analysis of the assembled and unassembled reads, a local BLAST database was set up in Geneious software using complete and partial fish mitochondrial genomes downloaded from the Mitochondrial Genome Database of Fish available at http://mitofish.aori.u-tokyo.ac.jp/download/. A local BLAST search was then performed on assembled (consensus sequences) and unassembled (unused) reads using the curated Fish Mitochondrial Genome Database in Geneious software with the following parameters: *Program: Megablast, Results: Hit table, Retrieve: Matching region with annotation, Maximum hits: 1, and Maximum E-value: 1e-1*. A sequence classifier database was created from the BLAST hits by removing duplicate sequences, downloading full hits, and extracting hit regions into a new folder. The extracted BLAST hits were batch renamed by replacing '*Name of Sequence*' with '*Organism*'. Sequence classification was performed on length filtered reads (150–220 bp) using the sequence classifier plugin in Geneious Software under the following parameters: *Sensitivity: High sensitivity/medium, Minimum overlap: 100 bp, Classify using taxonomy from: Database sequence taxonomy field, Minimum overlap identity to classify at lowest taxonomic level (e.g. species): 95 %, Minimum overlap identity to classify at second lowest taxonomic level (e.g. genus): 90 %, and Minimum overlap identity to classify at third lowest taxonomic level (e.g. family): 85 %.* Generated results, classification tables, were then exported into GraphPad Prism for further analysis.

Species diversity

Species diversity for each sampling site, as well as each stratum, was determined by using the Simpson Diversity Index (SDI). The SDIs were calculated using the formulae below:

$$D = 1 - \left(\frac{\sum n(n-1)}{N(N-1)}\right)$$

Where D – diversity
N – total number of individual organisms (all species combined) n – number of individuals of a particular species

Results

The Torrent Suite version 5.16.1 run of the pooled 12S, 16 amplicon libraries produced 32,983,264 reads (99.2 %, library Ion Sphere Particles - ISPs) as total biodiversity detected. After filtering polyclonal, low-quality reads, and adapter dimers, 10,237,191 reads (representing 31.0 %) were retained as final library ISPs. Sample E10 recorded the lowest reads at 429,306, and sample E3 had the highest at 946,128 (Tables 1 and 2; S1). The Simpson Diversity Index (SDI) showed varying scores ranging between 0 and 1. Scores close to one indicate high diversity whereas scores close to zero show low diversity. The SDIs for the three strata (I, II & IV) invaded by the invasive *Parachanna* species were found to be at >0.5. However, in stratum III, which recorded no trace of the invasive species, the SDI was the lowest at 0.41. Furthermore, three sites invaded by *Parachanna* (Mwatishi - E2, Chipita – E10 and Mulundu – E17) recorded

low SDIs of 0.26, 0.42 and 0.19, respectively. The lowest SDI was recorded in Puta – E5 (invaded by *Oreochromis niloticus*), with an SDI of 0.02. Luche lagoon -E13 and Lukwesa – E14 in stratum IV recorded the highest SDI at 0.81 (Table 1).

Correlation between eDNA read counts and species frequencies/diversity indices

Using the Statistical Package for the Social Sciences (SPSS), we evaluated the relationship between fish eDNA read counts and species frequencies/diversity indices of the sampled sites in the ML fishery. The coefficient associated between read counts and species frequencies ($r = 0.31$; p-value $= 0.239$) and diversity indices ($r = 0.1$; p-value $= 0.717$) were not statistically significant, indicating that read counts did not have a significant impact on species frequencies per sites and diversity indices. This result could be due to various factors, such as [24] non-inclusion of other variables in the model [15] non-linear or complex relationships between read counts and species frequencies/diversity indices and [29] small sample size to detect significant relationships (S3).

BLAST analysis – species composition

BLAST results showed a diversity of species in the samples sequenced. A total of 10 families (Cichlidae, Auchenoglanididae, Mormyridae, Clupeidae, Cyprinidae, Carangidae, Pangasiidae, Mochokidae, Clariidae and Channidae); three genera (*Clarias, Oreochromis and Parachanna*) and 13 species (*Tylochromis polylepis, Auchenoglanis occidentalis, Marcusenius senegalensis, Coptodon zillii, Oreochromis niloticus, Potamothrissa obtusirostris, Clypeobarbus pleuropholis, Gnathonemus petersii, Trachurus japonicus, Labeo nasus, Campylomormyrus compressirostris, Pangasianodon gigas and Synodontis schoutedeni* were detected. Only sequences with species names assigned with an overlap percent identity match >95 % on the MitoFish database were retained, except for *Parachanna*, which was matched to genus level at a low threshold (91.76–92.66 %). *Parachanna* was detected in sites with the highest or second to *Oreochromis* in frequency scores (Table 2).

A total of 15,546 species frequencies were detected in the 16 sampling sites of the four strata of the ML fishery. The highest frequency for families scored was Cichlidae ($n = 11{,}963$), followed by Channidae ($n = 3193$) and Mormyridae ($n = 167$), Auchenoglanididae ($n = 101$), Mochokidae ($n = 51$), Carangidae ($n = 29$) and Cyprinidae ($n = 24$). The lowest frequency at family level was recorded for Clupeidae ($n = 14$), Clariidae ($n = 3$) and Pangasiidae ($n = 1$). *Oreochromis* species produced the highest frequency ($n = 7267$), followed by invasive *Parachanna* species ($n = 3193$) and *Clarias* ($n = 3$). In descending order, the 13 species frequencies scored included *Tylochromis polylepis* ($n = 4388$), *Auchenoglanis occidentalis* ($n = 101$), *Marcusenius senegalensis* ($n = 71$), *Synodontis schoutedeni* ($n = 51$), *Coptodon zillii* ($n = 49$), *Trachurus japonicus* ($n = 29$), *Campylomormyrus compressirostris* ($n = 23$), *Labeo nasus* ($n = 19$), *Clypeobarbus pleuropholis* ($n = 5$), *Gnathonemus petersii* ($n = 2$) and a single score for *Oreochromis niloticus, Potamothrissa obtusirostris* and *Pangasianodon gigas* ($n = 1$). It is important to note that the common and indigenous *Oreochromis macrochir* was not detected.

Replicate frequencies

Parachanna species were detected in three (I, II and IV) of the four strata of the ML fishery, covering eight sites (Lunchinda, Kenani, Mwatishi, Kabuta, Chipita, Kashiba, Mulundu and Luapula River II) represented by samples, E1, E2, E3, E4, E10, E16, E17 and E18, respectively. The overlap percent identity ranged between 91.76 and 92.66 % (NC_022480), with frequencies of 13.24 %, 3.67 %, 3.44 %, 4.87 %, 3.67 %, 2.03 %, 6.06 % and 2.31 % for E1, E2, E3, E4, E10, E16, E17 and E18, respectively. *Oreochromis* was found to be present in all 16 sampling sites of the ML fishery, with a high abundance in stratum II, corresponding with a high abundance of the invasive species in the same stratum. The frequencies of *Parachanna* exceeded *Oreochromis* in three samples (E10, E16 and E17), in spite of being absent in Stratum III (Table 2; Fig 2a and b).

Species richness was detected with eDNA metabarcoding methods with relaxed protocols as indicated in the heat maps based on both strata and sampling sites. Heat maps were generated based on the overlap identity percentages and frequencies for species detected with eDNA metabarcoding as captured in Table 3. Heat maps also revealed the presence of another invasive species, *Oreochromis niloticus,* captured at Puta in stratum 1 of the ML fishery (Fig 3).

Discussion

This study aimed to investigate the presence and distribution of invasive *Parachanna, Oreochromis* and native species in the ML fishery using eDNA metabarcoding. It further applied the Simpson Diversity Index (SDI) for each sampling site and for each stratum to examine the diversity and relationship of species between impacted or invaded and non-impacted systems. Environmental DNA analysis detected a total of 10 families, three genera and 13 species, with the presence of *Oreochromis* species observed in all the 16 sites analysed. In contrast, the invasive *Parachanna* species was detected in eight sites. Furthermore, the SDI showed species diversity of >0.5 for the three invaded strata and low SDIs for the invaded individual sampling sites of 0.26, 0.42 and 0.19 for Mwatishi, Chipita and Mulundu, respectively. The lowest SDI of 0.02 was recorded at the Puta sampling site in stratum I invaded by invasive *Oreochromis niloticus* and five unexpected species (*Marcusenius senegalensis, Trachurus japonicus, Labeo nasus, Campylomormyrus compressirostris and Synodontis schoutedeni*) were also detected.

The non-sequencing of two pooled samples was due to low concentrations (two of the 18 pooled samples), similar to what Brys et al. [19] observed when the team detected seven out of the nine cage species from pooled water samples. This agrees with assertions propagated by Rees et al. [21] that the probability of detection of species is dependent upon taxa, type of aquatic ecosystem and concentration of the targeted species, whereby high eDNA decay rates and spatial dispersal can be among the attributes of poor or low

Table 1
Summary of read quality and species detected per sampling site and strata. Sampling strata and site, sample concentrations, bases, reads, mean read lengths, library concentrations and species frequencies per site and stratum.

Sample ID	Strata	Sampling site	Sample concentration (ng in µl)	Bases	≥ Q20	Reads	Mean read length (bp)	Library concentration (pM)	Species freq per site	Species freq per stratum	Simpson Diversity indices (SDIs) per site	SDI per stratum
E1	2	Kenani	88.72 in 27.5	112,189,027	99,174,390	491,436	228	14,364.1	2953		0.62	
E2	2	Mwatishi	29.00 in 15.0*	113,081,467	100,061,148	479,862	235	16,160.8	4009	11,162	0.26	0.59
E3	2	Kabuta	55.28 in 27.0	237,515,519	208,674,926	946,128	251	7063.0	4200		0.60	
E4	1	Lunchinda	48.32 in 10.5	120,904,841	106,637,977	483,137	250	15,211.0	933		0.60	
E5	1	Puta	52.68 in 55.0	178,439,261	157,905,883	664,801	268	12,007.7	1055	2121	0.02	0.60
E6	1	Kafulwe	72.32 in 13.5	137,204,770	121,036,516	515,277	266	13,398.8	133		0.37	
E7	3	Kilwa Islands	25.84 in 16.5*	134,582,227	119,342,338	509,279	264	16,392.7	212	212	0.41	0.41
E10	4	Chipita	16.48 in 11.0*	107,407,298	93,767,097	429,306	250	16,286.9	290		0.42	
E11	4	Luapula River I	83.20 in 11.0	137,470,791	121,904,402	500,689	274	15,688.8	48		0.42	
E12	4	Pembe lagoon	62.82 in 110	133,372,228	116,698,017	507,137	262	16,069.2	173		0.79	
E13	4	Luche lagoon	58.40 in 10.5	143,608,542	126,313,785	550,662	260	13,087.6	13	2051	0.81	0.65
E14	4	Lukwesa	68.24 in 33.0	168,028,367	147,842,848	631,132	266	6512.3	14		0.81	
E15	4	Kabula lagoon	86.56 in 27.0	186,850,241	165,479,615	685,626	272	9115.1	20		0.66	
E16	4	Kashiba	73.46 in 44.0	143,352,610	124,290,662	657,845	217	13,008.7	109		0.64	
E17	4	Mulundu	63.12 in 110	166,562,148	146,296,926	619,985	268	12,183.1	716		0.19	
E18	4	Luapula River II	79.20 in 27.5	159,142,789	138,501,241	636,373	250	8763.6	665		0.67	

* Below recommended sample concentration.

Table 2
Overlap identity percentages and frequencies for species detected. Sampling sites invaded by *Parachanna* species are denoted by highlighted yellow, including corresponding sequences from MitoFish reference database.

Species/Genus/Family	Overlap ID %	Sample identity																Closest sequence
		E1		E2		E3		E4		E5		E6		E7		E10		
		Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	
Tylochromis polylepis	95-100.00	561	8,83	81	0.89	2200	13.84	54	0.68	1044	6.16	105	1.07	159	1.45	38	0.65	NC_011171
Auchenoglanis occidentalis	95-98.94	1	0.02	70	0.77	6	0.04					1	0.01	3	0.03			NC_015809
Marcusenius senegalensis	95-100.00			5	0.06	2	0.01	1	0.01							2	0.03	NC_015090
Coptodon zillii	95-97.86			13	0.14	11	0.07	8	0.10			1	0.01			2	0.03	NC_026110
Potamothrissa obtusirostris	95-96.59					13	0.08							1	0.01			NC_016698
Clypeobarbus pleuropholis	95-95.60									3	0.02			2	0.02			NC_031627
Gnathonemus petersii	97-98.33																	NC_012717
Trachurus japonicus	99-99.47			3	0.03							14	0.14	8	0.07			NC_002813
Oreochromis niloticus	95.24									1	0.01							NC_013663
Labeo nasus	95-98.31																	NC_029449
Campylomormyrus compressirostris	98-98.95																	NC_022960
Pangasianodon gigas	95.15																	NC_006381
Synodontis schoutedeni	95-96.30											3	0.03	1	0.01			NC_015808
Clarias	98.32													3	0.03			NC_015749
Oreochromis	95-98.39	1509	23.76	3431	37.86	1356	8.53	438	5.51	7	0.04	6	0.06	35	0.32	33	0.56	NC_007231
Cichlidae	95-98.83	41	0.65	73	0.81	65	0.41	45	0.57			1	0.01					NC_018557
Mormyridae	95-99.40											2	0.02					NC_015086
*Parachanna**	91-92.66*	841	13.24	333	3.67	547	3.44	387	4.87							215	3.67	NC_022480

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Species/Genus/Family	Overlap ID %	Sample identity																Closest sequence
		E11		E12		E13		E14		E15		E16		E17		E18		
		Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	
Tylochromis polylepis	95-100.00	36	0.39	38	0.47	1	0.01	2	0.03	4	0.03	10	0.33	14	0.13	41	0.58	NC_011171
Auchenoglanis occidentalis	95-98.94							1	0.01			13	0.43	3	0.03	3	0.04	NC_015809
Marcusenius senegalensis	95-100.00	3	0.03	46	0.57											12	0.17	NC_015090
Coptodon zillii	95-97.86			1	0.01					1	0.01	2	0.07	1	0.01	9	0.13	NC_026110
Potamothrissa obtusirostris	95-96.59																	NC_016698
Clypeobarbus pleuropholis	95-95.60																	NC_031627
Gnathonemus petersii	97-98.33															2	0.03	NC_012717
Trachurus japonicus	99-99.47					4	0.05											NC_002813
Oreochromis niloticus	95.24																	NC_013663
Labeo nasus	95-98.31													2	0.02	17	0.24	NC_029449
Campylomormyrus compressirostris	98-98.95											1	0.03			22	0.31	NC_022960
Pangasianodon gigas	95.15					1	0.01											NC_006381
Synodontis schoutedeni	95-96.30			33	0.41	4	0.05	4	0.05	1	0.01			1	0.01	4	0.06	NC_015808
Clarias	98.32																	NC_015749
Oreochromis	95-98.39	7	0.08	18	0.22	3	0.04	5	0.07	11	0.08	18	0.60	49	0.46	341	4.82	NC_007231
+	95-98.83	2	0.02					1	0.01	3	0.02	2	0.07	2	0.02	23	0.33	NC_018557
Mormyridae	95-99.40			37	0.45			1	0.01			2	0.07	1	0.01	28	0.4	NC_015086
*Parachanna**	91-92.66*											61	2.03	646	6.06	163	2.31	NC_022480

detections [19]. De et al. [34] also attributed the non-amplification of markers to improper annealing temperatures arising from limited template DNA and PCR inhibitors. This can be attested by observing the wide range of annealing temperatures recorded in this study.

The species frequency of the invasive *Parachanna* species was found to be low, with a wide range of overlap identification percentages (91.76–92.66 %), as was illustrated by Antich et al. [8]. *Parachanna* species was found to be linked to *Parachanna insignis*, but not closely related. However, the identified gaps, coupled with the absence of 12S gene sequences of some *Parachanna* species deposited on GenBank, indicate a possible likelihood of sequences being closely related to either *P. obscura* or another new species of *Parachanna* as demonstrated by Conte-Grand et al. [32]. This was also in agreement with the findings by Doble et al. [22], who cited the non-availability of reference sequences for identifying rare and closely related species in tropical ecosystems.

From this study, it can be ascertained that the varying SDIs for various sites sampled cannot only be attributed to the impact of invasive *Parachanna* species as shown by the lowest score at Puta sampling site, which recorded another invasive, *Oreochromis niloticus*. The detection of *Oreochromis niloticus*, an elusive and not yet established species in the ML fishery, demonstrates the value of eDNA in the early detection of cryptic species that cannot easily be captured by conventional methods, as observed by Rees et al. [21]. The high SDIs recorded in the three impacted ML fishery strata have demonstrated invasive species' resilience even in diverse and severe environmental conditions, as shown by Davidson et al. [35]. To a lesser extent, low diversity indices in individual sampling sites could be attributed to low production rates and biomass.

Using the standard protocol, the detection rate was low, and no *Parachanna* species were detected from the eDNA water samples. However, with the relaxed protocol, detection rates improved except for samples collected from the interconnected Lagoons of the ML fishery. This observation of low detection rates in Lagoons, even with relaxed protocols, concurs with what Cantera et al. [14] stated when sampling in streams. Low detections of *Parachanna* in open water areas of the ML fishery agree with assertions by Kmentová et al. [36], where coastal areas were found to contain high species richness compared to open aquatic environments. In open aquatic environments, genetic material cannot be contained for more than 30 days without decay, as outlined by Dejean et al. [37]. Oosting et al. [38] also demonstrated the high levels of DNA degradation within 24 h when stored in ethanol. Furthermore, Riede [39], Ama-Abasi & Ogar [40], and Kpogue et al. [41] indicated that *Parachanna* species thrive in vegetative and muddy waters and not open waters. However, from this study, it could be assumed that the invasive species are not yet widespread, and their impact is minimal in all strata of the ML fishery at this stage.

The limited 12S database deposited on GenBank posed a challenge to comprehensively draw conclusions and understand the dynamics of freshwater species of the ML fishery. As highlighted by Ficetola et al. [20], such a challenge can be addressed by the use of multiple primers and metabarcodes to get a fuller understanding. This was also echoed by Doble et al. [22], who advocated for the need for adequate local reference databases and sequencing depth when studying diverse aquatic systems. As proposed by Sigsgaard et al. [3] on European weather loach, the present study recommends fully utilising the eDNA method alongside conventional methods to manage *Labeo altivelis* and other threatened or endangered species in the ML fishery. The use of this method by government departments and other institutions will enhance monitoring capabilities with minimal financial resources.

The non-sequencing of two pooled samples was due to low concentrations (two of the 18 pooled samples), which is similar to what

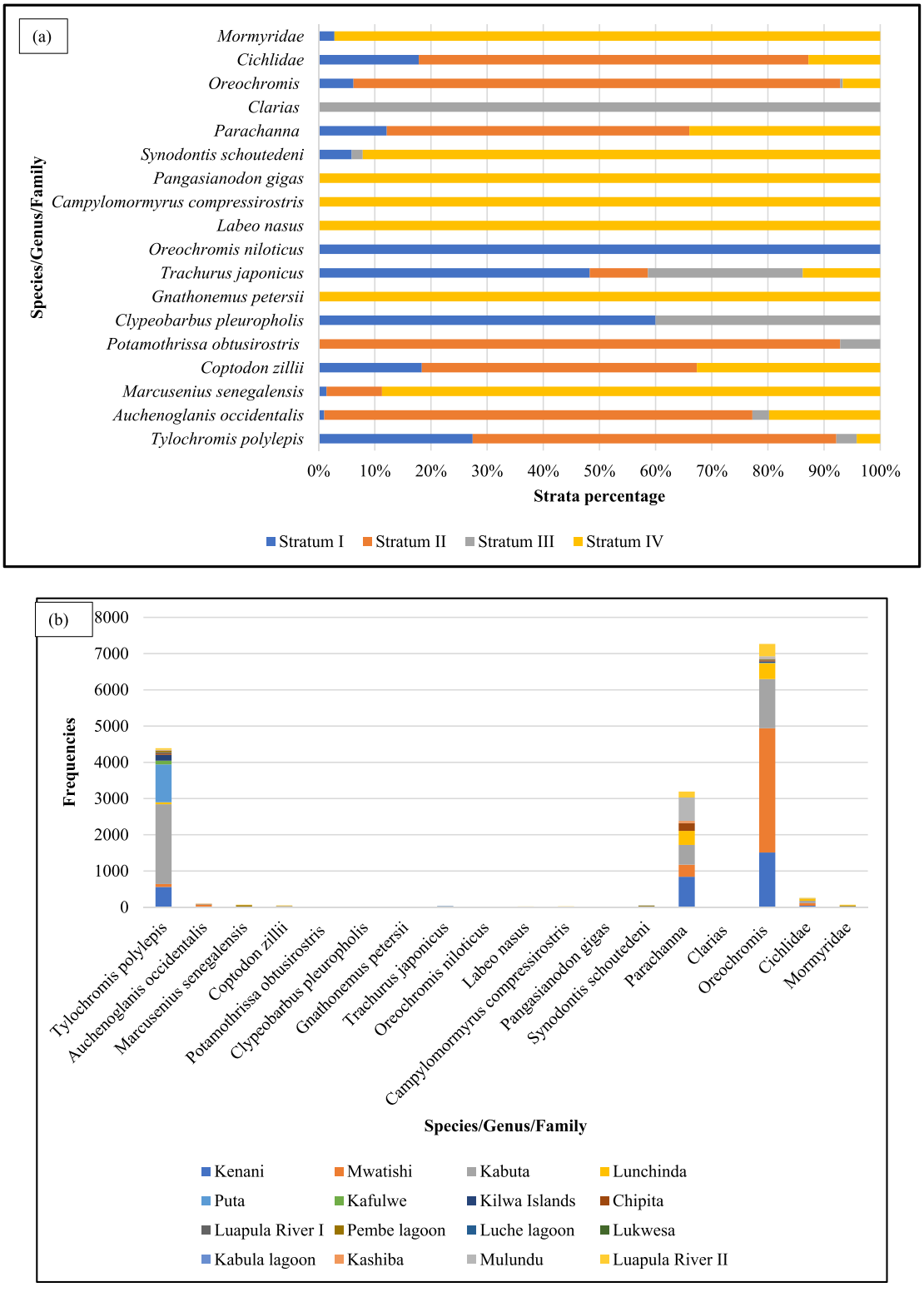

Fig. 2. (a) Species richness per stratum (b) Species richness per site detected with eDNA metabarcoding methods with an overlap percent identity match >95 % on MitoFish database.

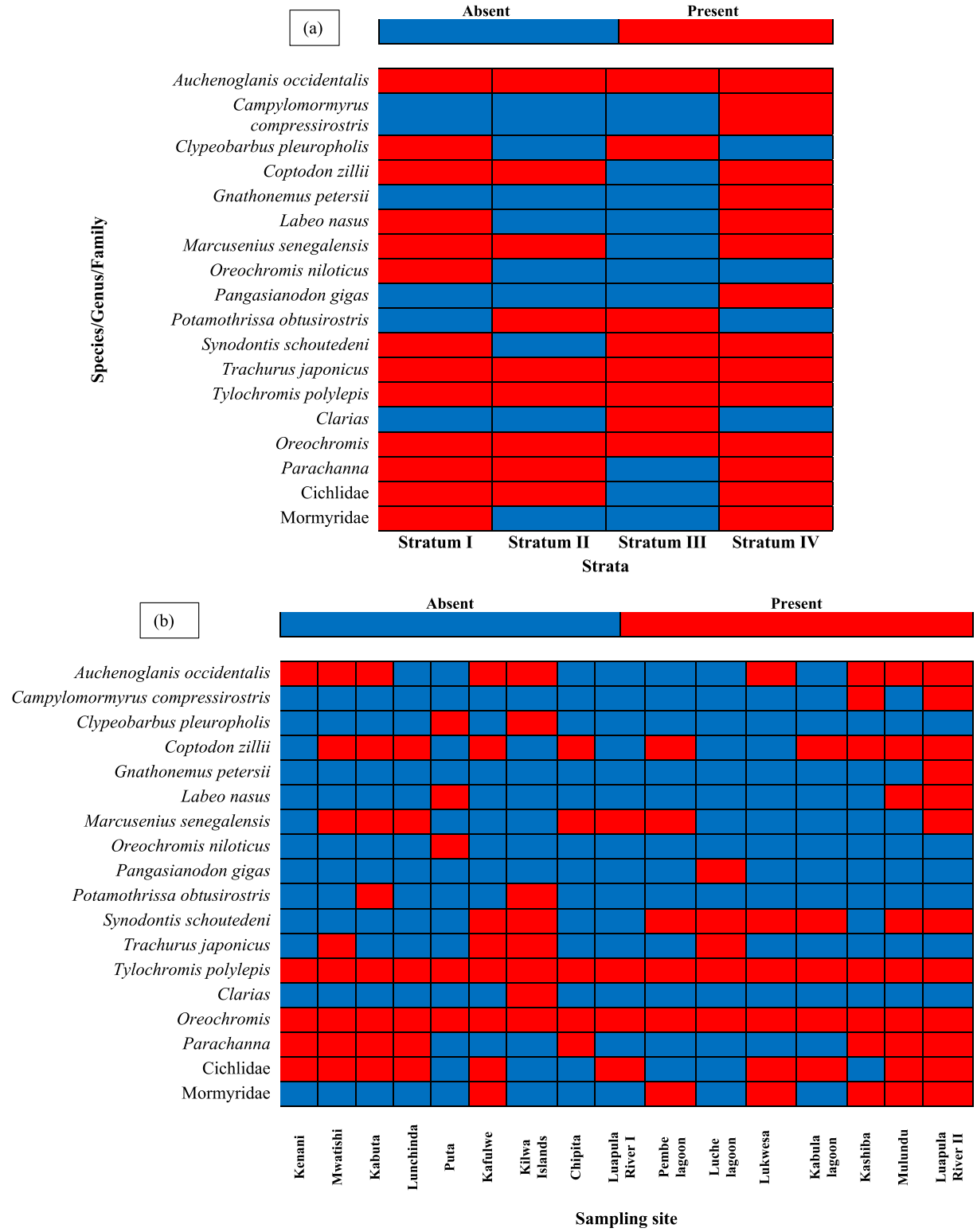

Fig. 3. Heatmap indicating the presence and absence of potential taxa by (a) strata and (b) sampling site for the 12S gene used for eDNA metabarcoding. Red rectangles indicate detection, and the blue rectangles show no detection.

Brys et al. [19] observed when the team detected seven out of the nine cage species from pooled water samples. This agrees with assertions propagated by Rees et al. [21] that the probability of detection of species is dependent upon taxa, type of aquatic ecosystem and concentration of the targeted species, whereby high eDNA decay rates and spatial dispersal can be among the attributes of poor or low detections [19]. De et al. [34] also attributed the non-amplification of markers to improper annealing temperatures arising from limited template DNA and PCR inhibitors. This can be attested by observing the wide range of annealing temperatures recorded in this study.

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Conclusion

The detection of five unexpected species (*Marcusenius senegalensis, Trachurus japonicus, Labeo nasus, Campylomormyrus compressirostris and Synodontis schoutedeni*) and the invasive *Parachanna* species in eight of the 16 sampled sites has demonstrated the essence of introducing the application of eDNA metabarcoding alongside traditional conventional methods in assessing species distribution and relative abundance in the ML fishery of Zambia. However, the study has exposed limitations in the 12S reference sequences for both *Parachanna* and *Oreochromis* species deposited in GenBank, which stood as a challenge in matching sequences to species level. Furthermore, the fluctuating SDIs have demonstrated the impact of invasive species at the sampling site level and not at the stratum level. However, the SDIs cannot be singularly attributed to the presence of invasive species, but other external factors are likely also at play. It remains important for future studies to encompass other factors such as illegal, unregulated, and unreported fishing methods and climate change as potential factors causing shifts in species composition in the ML fishery of Zambia.

Statement

All procedures were performed in compliance with relevant laws and institutional guidelines and have been approved by the appropriate institutional committee(s).

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CRediT authorship contribution statement

Bornwell Seemani: Conceptualization, Investigation, Methodology, Data curation, Visualization, Formal analysis, Funding acquisition, Writing – original draft, Resources, Project administration. **Carel Oosthuizen:** Supervision, Writing – review & editing, Data curation, Formal analysis, Project administration, Validation. **Cyprian Katongo:** Supervision, Writing – review & editing, Data curation, Formal analysis, Validation, Resources. **Arrie Klopper:** Supervision, Writing – review & editing, Data curation, Formal analysis, Validation. **Paulette Bloomer:** Supervision, Writing – review & editing, Data curation, Formal analysis, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sciaf.2025.e02544.

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