African Zoology

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/tafz20

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To cite this article: Mahlatse F Mashaphu, Gordon C O'Brien, Colleen T Downs & Sandi Willows-Munro (2023) The status of COI and 12S rRNA DNA barcode reference libraries for freshwater fish in South Africa: Implications for future eDNA projects, African Zoology, 58:3-4, 97-105, DOI: 10.1080/15627020.2023.2274334

To link to this article: https://doi.org/10.1080/15627020.2023.2274334

African Zoology 2023, 58(3–4): 97–105
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AFRICAN ZOOLOGY
ISSN 1562-7020 EISSN 2224-073X
https://doi.org/10.1080/15627020.2023.2274334

The status of COI and 12S rRNA DNA barcode reference libraries for freshwater fish in South Africa: Implications for future eDNA projects

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Environmental DNA metabarcoding (eDNA) is a rapidly emerging field in which high-throughput sequencing is used to catalogue the biodiversity of ecosystems through the amplification of DNA extracted from environmental samples (water, air, faeces and soil). Although eDNA has strong links to DNA barcoding, the molecular marker most often used to detect vertebrates in eDNA studies is a portion of the mitochondrial 12S ribosomal RNA (12S rRNA) and not the standard cytochrome oxidase I (COI) marker used in traditional DNA barcoding. eDNA methods rely on a comprehensive reference library to link sequence data to species, which are often lacking in hyper-diverse countries such as South Africa. In this study, we review the present state of DNA barcode reference databases for both 12S rRNA and COI for freshwater fish (native and introduced) found in South African aquatic systems. Analysis of DNA records available on GenBank and the Barcode of Life Database (BOLD) revealed incomplete records of the examined taxa for both markers. Our findings showed that 34 species, 6 genera and 0 families of native South African freshwater fish lack COI barcode records, while 86 species, 22 genera and 8 families lack 12S rRNA records. Unlike the native freshwater fish, the non-native fish all had barcode records available for both COI and 12S rRNA. Producing comprehensive reference libraries for both markers is an important first step in developing an eDNA protocol for the non-invasive monitoring of native and non-native freshwater fish in South Africa.

Keywords: biomonitoring, COI gene, DNA barcoding, metabarcoding, 12S rRNA gene

Supplementary material: available at https://doi.org/10.1080/15627020.2023.2274334

Introduction

DNA barcoding has accelerated species identification and has been used to monitor changes in species composition in ecosystems (Hebert et al. 2003; da Silva and Willows-Munro 2016; Elsaied et al. 2021; Singh et al. 2021). Metabarcoding extends DNA barcoding by using high-throughput sequencing technology to allow for rapid production of species inventories from complex bulk samples (Singh et al. 2021). Environmental DNA (eDNA) uses DNA extracted from environmental samples such as soil, air, or water (Taberlet et al. 2012; Belle et al. 2019) and provides the opportunity for non-invasive sampling and monitoring (Miya et al. 2015; Thomsen and Willersleve 2015; Valentini et al. 2016; Belle et al. 2019; Alam et al. 2020; Keck et al. 2022). In particular, many studies have demonstrated the utility of eDNA in monitoring species linked to aquatic systems (Hänfling et al. 2016; Vasselon et al. 2017; Fernández et al. 2018; Mächler et al. 2019; Keck et al. 2022; among others). Despite the promise of providing important global baseline data on species distribution and abundance essential for conservation and management (Heywood 2011; Belle et al. 2019), eDNA research in African systems is still limited (Belle et al. 2019).

Globally, freshwater ecosystems are particularly vulnerable to multiple stressors derived from anthropogenic activities (Revenga et al. 2005; Dudgeon 2010; Dudgeon 2019; Belle et al. 2019; Fierro et al. 2019; Reid et al. 2019; Alam et al. 2020). This is particularly true in South Africa, which is a water-scarce country (Dallas and Rivers-Moore 2014; Govender et al. 2022). Freshwater ecosystems in South Africa are species-rich (Dudgeon 2019; O'Brien et al. 2019; Dallas et al. 2022), with high levels of endemism (Ellender et al. 2017; Dallas et al. 2022) and are affected by factors including pollution, water extraction, the introduction of invasive species and the overexploitation of aquatic resources (Dudgeon et al. 2006; Dallas and Rivers-Moore 2014; Riddell et al. 2019; Adams et al. 2020; Desai et al. 2021; Dallas et al. 2022; Evans et al. 2022). These activities pose a major threat to the freshwater biodiversity in the region (Dallas and Rivers-Moore 2014; O'Brien et al. 2019; Desai et al. 2021; Dallas et al. 2022). There is an ever-increasing need to effectively monitor changes in biodiversity, identify the most affected areas and establish priority conservation areas for vulnerable taxa. Species identification, discovery and monitoring have become an essential research theme for the conservation and management of biodiversity (Tsoupas et al. 2022). Sustainable conservation of freshwater biodiversity requires baseline knowledge of the community structure of natural ecosystems (Fierro

et al. 2019). This will aid in understanding the impact of anthropogenic and natural activities on biodiversity loss (Fierro et al. 2019; Desai et al. 2021).

Environmental DNA could provide an important tool for monitoring biodiversity in aquatic systems in South Africa (and other countries). Future eDNA research may be held back by the lack of comprehensive DNA reference libraries linking DNA barcodes to taxonomically verified reference (voucher) specimens (Elbrecht et al. 2017; Leese et al. 2018; Weigand et al. 2019; Garcia de Amézaga Quintanilla 2021; Singh et al. 2021; Li et al. 2022). The molecular marker in the DNA barcode community most widely used for identifying animal taxa is the standardised 658-base pair (bp) portion of the cytochrome C oxidase subunit I gene (COI; Hebert et al. 2003). These data are curated mainly in two popular databases, the Barcode of Life Database (BOLD; Ratnasingham and Hebert 2007) and GenBank. In contrast, recent eDNA metabarcoding studies have relied on the 12S ribosomal mitochondrial gene (12S rRNA) for the detection of vertebrate taxa (Riaz et al. 2011; Kelly et al. 2014; Miya et al. 2015; Hänfling et al. 2016; Yamamoto et al. 2017; Polanco et al. 2021), including the characterisation of fish communities in freshwater habitats (Thomsen et al. 2012; Evans et al. 2016; Valentini et al. 2016; Bylemans et al. 2018; Cilleros et al. 2019; Fujii et al. 2019; Lecaudey et al. 2019; Berger et al. 2020; Antognazza et al. 2021; Hallam et al. 2021; Sales et al. 2021; García-Machado et al. 2022). As eDNA research for biodiversity monitoring is still being established in South Africa, this review aimed to summarise the available DNA barcode reference libraries for freshwater fish (both native and introduced). Specifically, we compared the COI and 12S rRNA data available for fish found in South African freshwaters and make some suggestions for the standardisation of techniques used in future aquatic eDNA research.

Materials and methods

Our review of available 12S rRNA and COI records focused on all current native freshwater fish; we only considered primary freshwater fish that are restricted to and complete their life cycle in freshwater (Myers 1938). We collated a list of native freshwater fish using Skelton (2001), Chakona et al. (2022) and FishBase (Froese and Pauly 2023). Our review also included introduced freshwater fish that have naturalised in South African freshwaters (Ellender and Weyl 2014; Weyl et al. 2020). We compiled a list of introduced fish species from Weyl et al. (2020) and FishBase (Froese and Pauly 2023). We checked the availability of COI and 12S rRNA sequence data for each species by searching the BOLD (Ratnasingham and Hebert 2007) and National Centre for Biotechnology Information (NCBI, GenBank) databases. Our study included all data available up to and including October 2023. Given that eDNA often makes use of short-read high-throughput sequencing technologies such as Illumina, we noted all COI and 12S rRNA sequences >300 bp as present in the database. We also considered the availability of genes from both whole genomes and mitogenomes. Where possible, we also noted if the reference individual was collected in South Africa or another country. This was only noted if the country of collection was reported for the sequences in both BOLD and GenBank. If the barcode specimen was collected outside of South Africa, we still considered the record available for that species.

Results

The species list that we compiled included 106 native South African freshwater fish species (37 genera and 17 families; Supplementary Table S1) and 20 non-native species (15 genera and seven families; Supplementary Table S2). The alien species were introduced to South African aquatic systems through release from the pet trade, for recreational fishing, or for aquaculture (Ellender and Weyl 2014; Weyl et al. 2020). For native fish, only 72 COI records were available, representing only 65% of the native species found in South Africa. Of these, only 47 records (65%) were collected from localities in South Africa, and 42 (58%) were full-length (>600 bp) COI barcodes (Table 1). At higher taxonomic levels, 84% of native fish genera had at least one COI record, while all (100%) families were represented by at least one record. For 12S rRNA, only 20 (19%) native species, 15 genera (41%) and nine families (53%) were represented by at least one record (Table 1). All the 12S rRNA data (100%) were sequenced from individuals collected outside of South Africa. Of the 20 non-native freshwater species found in South Africa, all had COI and 12S rRNA barcode records. Of these records, only 12 (60%) COI records were from specimens collected in South Africa (Table 1). Consequently, the non-native species were also fully represented at higher taxonomic levels, with 100% of genera and families covered for both COI and 12S rRNA (Table 1).

Not all the examined genera of South African freshwater fish had available COI or 12S rRNA barcode records. The genera *Ctenopoma, Microctenopoma, Serranochromis, Amatolacypris, Namaquacypris* and *Silhouettea* lacked barcode records for both markers (Figure 1a). Among the native freshwater fish families found in South Africa, both COI and 12S rRNA records were available for nine families, while the remaining eight families had records for only COI (Figure 1b). For non-native freshwater fish in South Africa, all the genera and families had barcode records for both markers (Figure 2).

Given that not all the South African freshwater fish genera and families we examined contained the same number of species, we also present the data as a proportion of coverage (% of species with records in each genus and family). Among the native South African freshwater fish genera, *Pseudobarbus* followed by *Labeo* had the lowest proportion coverage for COI, while *Enteromius* followed by *Labeo* and *Amphilius* had the lowest proportion coverage for 12S rRNA (Supplementary Figure S3a). Among the various families of native South African freshwater fish, the lowest proportion of coverage for COI was recorded in the Anabantidae family, followed by the Gobiidae family (Supplementary Figure S3b). The lowest proportion coverage for 12S rRNA was found in the Cyprinidae family, followed by the Amphiliidae family (Supplementary Figure S3b). For non-native freshwater fish in South Africa, there was complete proportion coverage (100%) for COI and 12S rRNA barcode records for all genera and families (Supplementary Figure S4).

Table 1: Number of COI and 12S rRNA records available for native and introduced freshwater fish in South Africa (SA). The numbers of records for each species, genus and family are presented. We distinguished between records from specimens collected in South Africa and those only available from specimens collected outside the borders of South Africa

Taxonomy	Total in SA	Total COI records	COI records <600 bp	SA COI records	COI records from other countries	Total 12S rRNA records	12S rRNA records <600 bp	SA 12S rRNA records	12S rRNA records from other countries
Native taxa									
Species	106	72	31	47	25	20	4	0	20
Genus	37	31	7	21	10	15	5	0	15
Family	17	17	2	12	5	9	2	0	9
Introduced taxa									
Species	20	20	2	12	8	20	10	0	20
Genus	15	15	0	9	11	15	8	0	15
Family	7	7	0	6	1	7	1	0	7

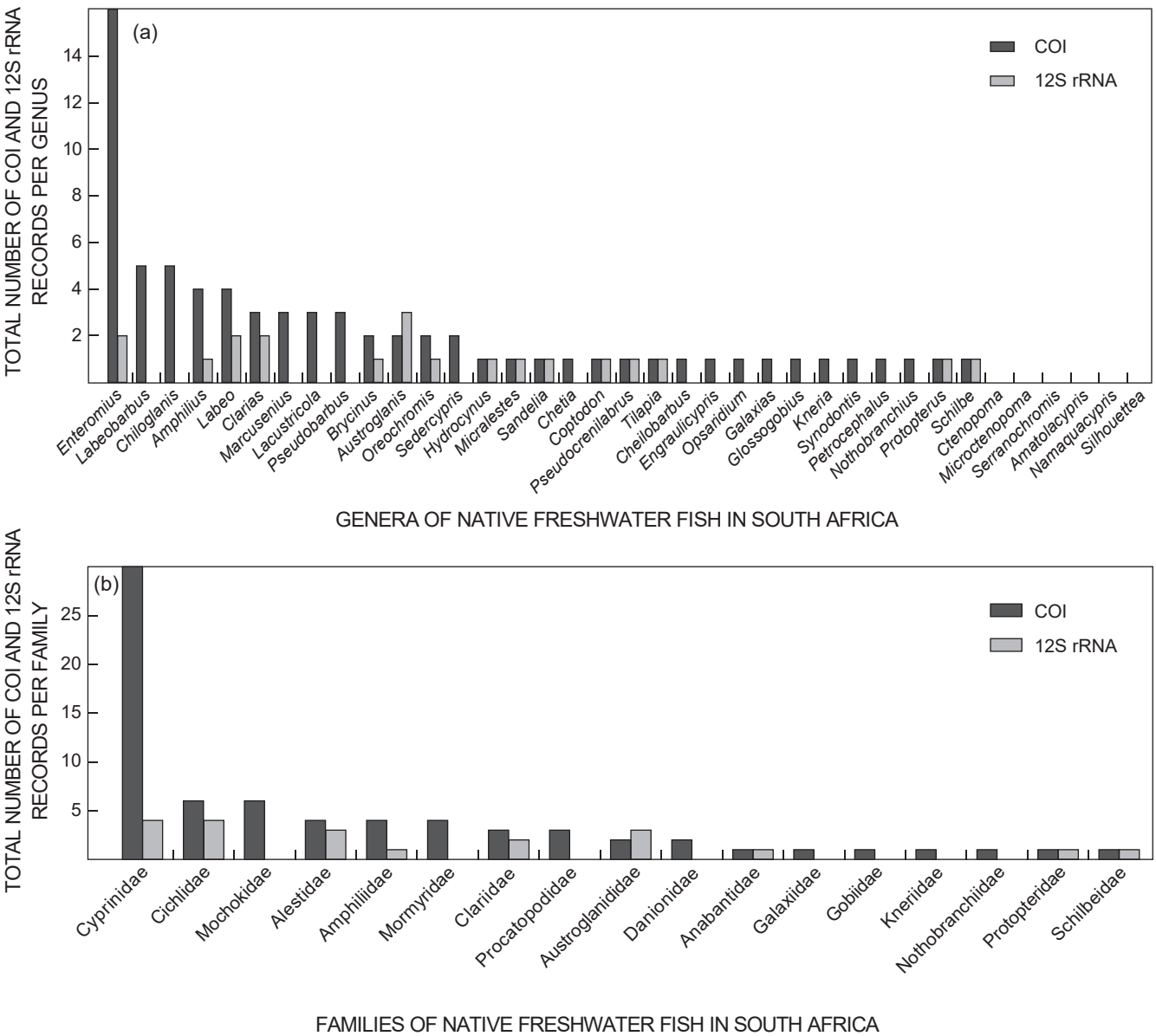

Figure 1: Total number of native freshwater fish species with (a) COI and 12S rRNA records per genus (genera are ranked according to the number of COI data records) and (b) COI and 12S rRNA records per family (families are ranked according to the number of COI data records)

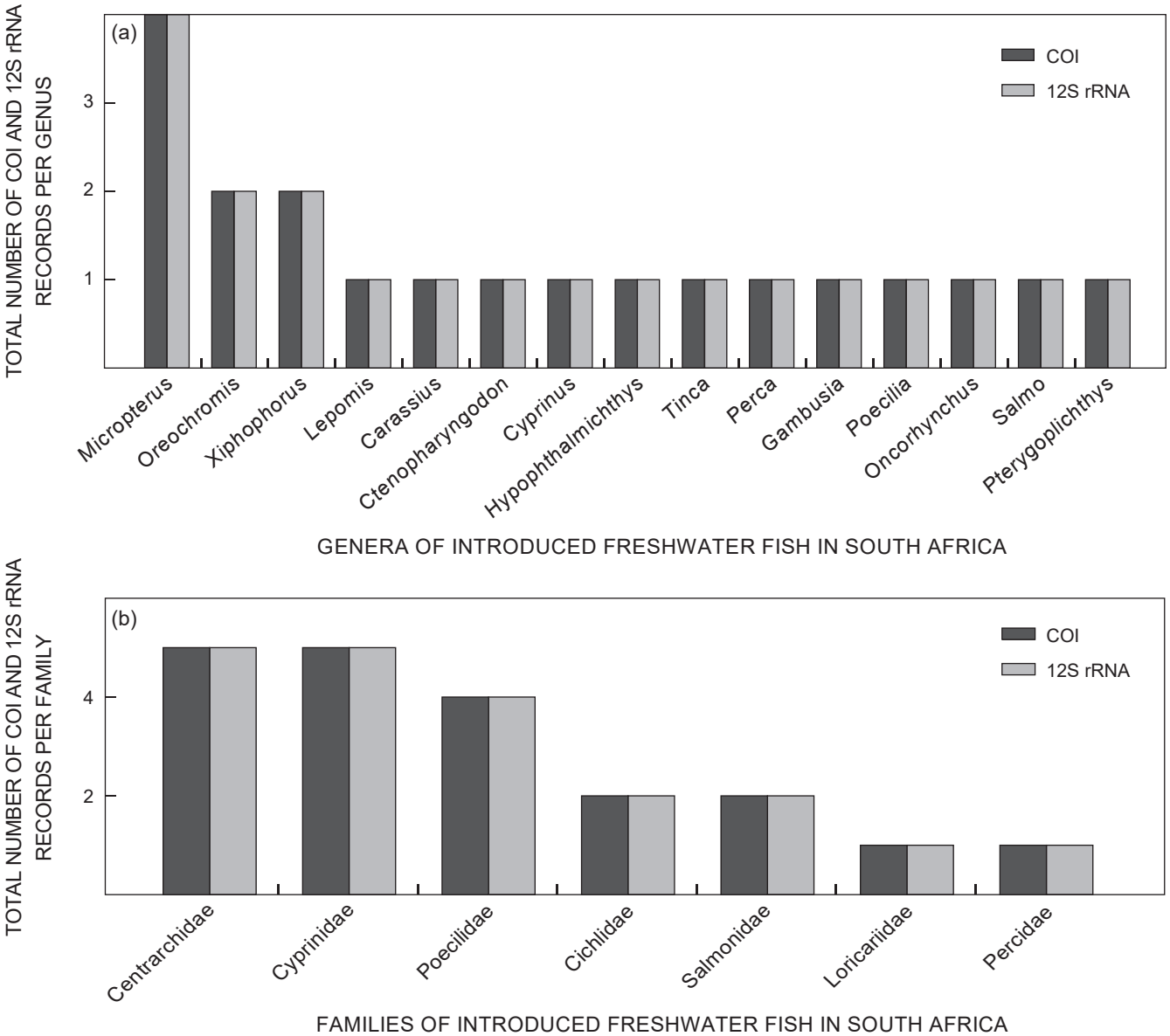

Figure 2: Total number of introduced freshwater fish species with (a) COI and 12S rRNA records per genus (genera are ranked according to number of COI data records) and (b) COI and 12S rRNA records per family (families are ranked according to the number of COI data records).

Discussion

This study reviewed the present state of COI and 12S rRNA DNA barcode reference libraries for native and introduced freshwater fish in South Africa. Although COI is the marker traditionally used in the DNA barcoding of animal species (Hebert et al. 2003; Bucklin et al. 2011; Leray et al. 2019; Li et al. 2022), 12S rRNA has recently been used in eDNA studies in other countries (Thomsen et al. 2012; Kelly et al. 2014; Miya et al. 2015; Noble et al. 2015; Evans et al. 2016; Valentini et al. 2016; Bylemans et al. 2018; Milan et al. 2020; Shu et al. 2021; Polanco et al. 2021; Xiong et al. 2022). This review highlights the incompleteness of the DNA reference libraries for South African native fish species for both COI and 12S rRNA sequences; in particular, the 12S rRNA DNA barcode reference library is poorly populated. The South African node of the International Barcode of Life (http://www.ibolproject.org) was established in 2010 and has been coordinating research efforts leading to significant growth of the COI DNA sequence reference library for South African taxa (da Silva and Willows-Munro 2016). This has likely led to the higher number of COI records than 12S rRNA records, despite the 12S rRNA marker becoming an increasingly important gene for the identification and monitoring of fish using eDNA methods (Deagle et al. 2014; Collins et al. 2019; Zhang et al. 2020; Polanco et al. 2021). For future eDNA studies in South Africa to be comparable to those conducted in other parts of the globe, this review suggests that a multi-marker approach (using both COI and 12S rRNA) be used. Moreover, using other genes, such as 12S rRNA and 16S rRNA, in addition to COI will allow

for more accurate species assignment and the elucidation of phylogenetic relationships at higher taxonomic levels (Duarte et al. 2020; Ahmed et al. 2022). To this end, it is also essential that the present 12S rRNA reference library for freshwater fish in South Africa be improved, particularly for native species. The DNA barcode reference libraries for the Cyprinidae family, which represents an important component of the freshwater fish in South Africa, are still lacking. The Cyprinidae family contains 56 species belonging to eight genera, of which 18 species are threatened (six Vulnerable, nine Endangered and three Critically Endangered). In particular, the *Pseudobarbus* genus is the most threatened in South Africa, with most species in this genus listed as Endangered or Critically Endangered on the IUCN red list (Chakona et al. 2022). The findings of this review indicate that most of these threatened species are not present in the current reference libraries for either COI or 12S rRNA DNA sequences. Of the 18 threatened species belonging to the Cyprinidae family, only three Vulnerable species (*Enteromius anoplus s.s.*, *Pseudobarbus swartzi* and *Pseudobarbus burgi*), and two Critically Endangered species (*Enteromius treurensis* and *Sedercypris erubescens*) have COI barcode records, and there are no 12S rRNA records available. Nevertheless, genes such as cytochrome *b* (cyt*b*), which has also been used in fish species identification (Tobe et al 2009; Ficetola et al. 2010), are becoming increasingly popular in eDNA research (Rees et al. 2015; Shu et al. 2020; 2021). One advantage of including cyt*b* in a multi-marker panel for eDNA, is that the substitution rate of this marker could provide support for higher taxonomic associations (Gillet et al. 2018). Although this review highlights the incompleteness of the 12S rRNA sequence reference library, we also reviewed the Cyt*b* sequence reference library (results not presented in this review) and it is more complete, with records available for 65 species, representing 61% compared to the 19% representation reported for 12S rRNA in this review. Furthermore, although the *Pseudobarbus* genus lacks COI and 12S rRNA barcodes, almost all the species belonging to this genus have Cyt*b* records available in GenBank. This further highlights the importance of using multi-marker approaches, which include the use of genes such as Cytb that have more complete reference libraries, for eDNA studies in South Africa.

Environmental DNA methods have also been used successfully for the detection and monitoring of invasive fish (Takahara et al. 2013; Keskin 2014; Bylemans et al. 2016; Keskin et al. 2016; Hinlo et al. 2017; Clusa and García-Vázquez 2018; Jo et al. 2021; Minett et al. 2021; Dubreuil et al. 2022; Jeunen et al. 2022). Considerable efforts have been made to barcode introduced freshwater fish species in South Africa (van der Walt et al. 2017). As a result, introduced freshwater fish species in South Africa are fully represented (100%) in the reference libraries for both COI and 12S rRNA DNA sequences, and this may promote the use of eDNA metabarcoding for the early warning, detection, monitoring and management of these introduced species.

The identification of taxa using DNA-based approaches also depends on the geographical coverage of local species in barcode reference databases (Li et al. 2022). This has been shown to improve species assignment by increasing taxonomic resolution during sequencing (Singh et al. 2021). According to Jones et al. (2021), complete DNA barcoding databases for regions or countries are still scarce. An important finding from this review is that most COI data (65%) for native freshwater fish were from specimens collected in South Africa. In contrast, all the 12S rRNA barcodes were from specimens collected outside the borders of South Africa (100%). This further highlights the need to build the 12S rRNA barcode reference library for South Africa to improve taxonomic resolution during eDNA analyses.

Our review suggests that gaps in the reference libraries for COI and 12S rRNA sequences will negatively affect the use of eDNA metabarcoding to monitor freshwater fish in South Africa. Therefore, priority should be given to filling these gaps, especially at the species level, as this could increase the efficiency and accuracy of species assignment (Duarte et al. 2020). However, we suggest that best practices for building reference libraries be employed for both genes. This will guarantee the best possible quality and traceability of the supporting information linked to the identification reference barcode. Although, BOLD (Ratnasingham and Hebert 2007) and GenBank (Benson et al. 2012) are the main repositories of DNA barcodes, they have been associated with species misidentification attributed to a lack of expert taxonomic verification and supporting information linked to the barcodes (Meiklejohn et al. 2019; Weigand et al. 2019; Rimet et al. 2021), with particular emphasis on the limitations of GenBank (Meiklejohn et al. 2019). According to Remit et al. (2021), a barcode sequence can only be considered reliable if its metadata are available, including the primary data and all supporting information for that DNA barcode. This includes the accurately identified voucher specimen, photographs, taxonomic name, collection location, storage facility information and barcode authors (Rimet et al. 2021). These practices should be observed for building high quality and reliable COI and 12S rRNA DNA barcode reference libraries for South African freshwater fish, which will enable efficiency when employing eDNA methods.

Conclusions and recommendations

Species discovery, identification, biodiversity monitoring and management are important measures for assessing the impacts of ecosystem management, climate change, habitat degradation, and other anthropogenic stressors and impacts on freshwater biodiversity in South Africa. Environmental DNA metabarcoding provides an opportunity for non-invasive monitoring and the identification of both native and introduced fish in freshwater systems. Despite this, the technique has not been established in South African inland waters and our study provides the initial step in the development of an eDNA metabarcoding protocol for monitoring freshwater fish in South Africa.

This review assessed the status of the DNA barcode reference libraries of the two main eDNA metabarcoding markers (COI and 12S rRNA) for native and introduced freshwater fish in South Africa. Our results highlighted the incomplete representation and coverage of indigenous

species in the barcode reference libraries for COI sequences and particularly for 12S rRNA. These gaps limit the use of eDNA metabarcoding technologies for discovering and managing these species in the region. Therefore, there is an urgent need to build reliable DNA barcode reference libraries for both markers for South African freshwater fish. The present state of the South African DNA barcode libraries provides the impetus to coordinate ongoing efforts and stimulate new initiatives aimed to fill the gaps in the barcode libraries for freshwater fish in South Africa. eDNA methods are innovative, robust and effective, and are contributing to sustainable water resource management and conservation globally. We have the same opportunities for this approach to contribute to South African freshwater research. This review identifies the foundational data needed to achieve this.

Acknowledgments — We are grateful to the University of KwaZulu-Natal (South Africa) and the National Research Foundation (South Africa, Grant 98404) for funding.

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Manuscript received: 1 December 2022, revised: 25 April 2023, accepted: 19 October 2023
Associate Editor: A Chakona