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Comparing the performance of 12S mitochondrial primers for fish environmental DNA across ecosystems

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Abstract

Through the development of environmental DNA (eDNA) metabarcoding, in situ monitoring of organisms is becoming easier and promises a revolution in our approaches to detect changes in biodiversity over space and time. A cornerstone of eDNA approach is the development of primer pairs that allow amplifying the DNA of specific taxonomic groups, which is then used to link the DNA sequence to taxonomic identification. Here, we propose a framework for comparing primer pairs regarding (a) their capacity to bind and amplify a broad coverage of species within the target clade using in silico PCR, (b) their capacity to not only discriminate between species but also genera or families, and (c) their in situ specificity and efficiency across a variety of environments. As a case study, we focus on two mitochondrial 12S primer pairs, MiFish-U and teleo, which were designed to amplify fishes. We found that the performance of in silico PCRs were high for both primer pairs, but teleo amplified more genera across Actinopterygii, Chondrichthyes, and Petromyzontomorphi than MiFish-U. In contrast, the discriminatory power for species, genera, and families were higher for MiFish-U than teleo, likely associated with the greater length of the amplified DNA fragments. The evaluation of their in situ efficiency showed a higher recovered species richness of teleo compared to MiFish-U in tropical and temperate freshwater environments,

but that generally both teleo and MiFish-U primers pairs perform well to monitor fish species. Since more species were detected when used together, those primer pairs are best used in combination to increase the ability of species detection.

KEYWORDS
biodiversity, biomonitoring, environmental DNA, fishes, in silico PCR, neural network

1 | INTRODUCTION

Biomonitoring tools are becoming increasingly necessary for monitoring the dynamics of biodiversity, especially in the light of global changes, which are causing a rapid species population decline (Kindsvater et al., 2014). Biomonitoring should go beyond quantification of the most abundant species and monitor entire assemblages, including rare species that can play an important role in ecosystems (Mouillot et al., 2013). Traditional wildlife census methods are generally not sufficient on their own, because they often overlook hard-to-monitor taxonomic groups. Moreover, the extensive time and financial costs of implementing these methods often limit the number of studied sites (Ficetola et al., 2019; Pawlowski et al., 2020; Plaisance et al., 2011). Another issue is the destructive sampling techniques of traditional capture methods in which organisms are often harmed due to the use of traps, nets, and even toxins (Murphy & Willis, 1996). The breakthrough in the ability to recover DNA from environmental samples (eDNA) using metabarcoding technology has led to an easier, cheaper, faster, and noninvasive method of detecting and monitoring organisms (Cordier et al., 2020; Taberlet et al., 2012; Yamamoto et al., 2017). This method allows for the identification of species composition using predesigned molecular primers that target specific clades (Deiner et al., 2017). The recovered sequences can then be compared with a genetic reference database and used to identify the taxa present in the environmental samples (Valentini et al., 2016). The application of eDNA metabarcoding increases the species detectability and permits more exhaustive ecosystem monitoring compared to previous approaches (Cilleros et al., 2019). eDNA metabarcoding was shown to be particularly adequate to monitor fish species in environments where they are difficult to detect, like in large rivers (Blackman et al., 2021; Cantera et al., 2019; Pont et al., 2018), lakes (Fujii et al., 2019; Hänfling et al., 2016; Lacoursière-Roussel et al., 2016) or marine ecosystems (Boussarie et al., 2018; Polanco Fernandez et al., 2021; West et al., 2020). Because the development of eDNA metabarcoding is relatively recent (Deiner et al., 2017), its application for global conservation of biodiversity should be accompanied by further technical performance assessments.

eDNA monitoring should provide a general solution applicable to a variety of ecosystems (e.g., freshwater, brackish, and marine) for conservation and management applications by recovering a broad phylogenetic diversity within the target clade and increasing the detection of rare species that support important and vulnerable functions (Mouillot et al., 2013). To achieve these ambitions, a cornerstone of the application of eDNA metabarcoding is the development of primer pairs for DNA amplification through polymerase chain reaction (PCR) (Collins et al., 2019; Leese et al., 2021; Zhang et al., 2020). Associated with the increased use of eDNA metabarcoding, a large variety of primer pairs have been developed, either universally (Bagley et al., 2019; Stat et al., 2019) or specifically to amplify target clades (Kress et al., 2015; Xia et al., 2018). To test their efficiency, computational tools reproducing in silico PCR have been developed to simulate potential amplification of primers across a species sequence database. Primer pairs are most frequently evaluated in silico for their capacity to bind and amplify a broad range of species (Cannon et al., 2016; Valentini et al., 2016), and their ability to discriminate among species within the target clade (Bylemans et al., 2018). In silico comparisons of eDNA primer pairs, either from the same or distinct regions of the genome, have generally shown varying levels of performance (Collins et al., 2019; Zhang et al., 2020) and can have many pitfalls (So et al., 2020). In contrast to in situ performance, in silico PCR amplifications can be overoptimistic. Specifically, abiotic and biotic conditions of the studied ecosystem could have an influence on the realized performance (Robson et al., 2016). In order to allow widespread application of eDNA metabarcoding for aquatic biodiversity monitoring, systematic performance assessments of primer pairs should combine both in silico and in situ under varied conditions (So et al., 2020; Zhang et al., 2020).

An important feature of well-performing primer pairs is the ability to not only recover the species through the species-specificity of the targeted sequence (Wilcox et al., 2013; Zhang et al., 2020), but also assign recovered eDNA sequences to higher taxonomic levels if a more precise taxonomic resolution cannot be guaranteed, or in the absence of a high-quality reference database. Generally, the number of nucleotide differences between DNA sequences of different species amplified by primer pairs represent a good proxy of the species specificity of the amplified region. A species can be considered unambiguously assigned if the marker sequence differs from the marker sequences associated with all other species by at least one base pair, regardless of the marker size (Boyer et al., 2016). Nevertheless, the main limitation of species assignment is the incomplete coverage of reference databases (Marques et al., 2020). While an international effort has led to the compilation of a large cytochrome oxidase 1 data base (Weigand et al., 2019), this region of the mitochondrial genome has revealed a low performance as an eDNA target region for some organismal groups, such as vertebrates, in proximity with variable regions allowing for species-level discrimination (Collins et al., 2019). For taxonomic groups other than invertebrates, other regions such as the 12S and 16S ribosomal RNA (rRNA) have shown higher performance in discriminating between organisms and consequently, new primer pairs have been

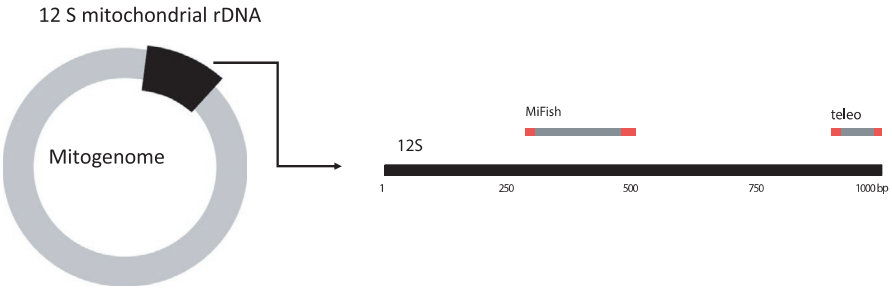

FIGURE 1 Schematic representation of (a) the mitochondrial mitogenome with the 12S mitochondrial rRNA gene and (b) the position and length of the metabarcode associated with the corresponding primer pair: MiFish-U and teleo

developed (Collins et al., 2019; Komai et al., 2019; Pawlowski et al., 2012). However, a major weakness of the use of those alternative primer pairs is the limited taxonomic coverage of the corresponding reference data base, where many unique sequences cannot be properly assigned to the species level (Collins et al., 2019). In the absence of complete references, it is therefore relevant to at least be able to classify reads to higher taxonomic levels (genera or family). To explore whether reads can be assigned to higher taxonomic levels, Lower Common Ancestor algorithms allow for a systematic comparison of sequences to the reference data base across taxonomic levels and assign the category with the highest level of confidence (Krause et al., 2008). Because the alignment and subsequent analyses of short, highly variable and noncoding DNA sequences are computationally challenging, an alternative method is to train neural networks to classify eDNA sequences into taxonomic classes based on motifs learned from independent DNA sequences. Neural networks have been shown to perform well for taxonomic assignments of microbial sequences based on patterns within the DNA sequence (Busia et al., 2019) and can help test taxonomic assignment abilities at various taxonomic levels. Generally, the flexibility of neural networks and their ability to learn complex patterns from large numbers of short sequences make them a promising choice for solving the general sequence-labeling problem in eDNA research (Nugent & Adamowicz, 2020).

Fishes are excellent bioindicators of ecosystem health (Chovanec et al., 2003; Fierro et al., 2017) and fulfil many ecosystem services (Holmlund & Hammer, 1999), which led to the development of multiple primer pairs to monitor their diversity and composition within freshwater (Cilleros et al., 2019; Milhau et al., 2019) and marine environments (Nguyen et al., 2020; Polanco Fernandez et al., 2021). Most studies have conducted eDNA surveys of fish assemblages using a single set of primers without prior evaluation of potential bias for the studied taxonomic group or ecosystem (e.g., Balasingham et al., 2017; Kelly et al., 2014). Multiprimers comparison generally found considerable differences in the amplified taxonomic specificity and species discrimination power both in silico and in situ (Bylemans et al., 2018; Zhang et al., 2020). For example, Bylemans et al., (2018) showed that among multiple primer pair comparisons, three primer pairs on the mitochondrial 12S rRNA gene (MiFish-U/teleo/AcMDB07) had the highest performance to recover fish species richness from rivers in Australia. Similarly, Zhang et al., (2020) showed that the two longest primer pairs of the 12S region Ac12S (Evans et al., 2016) and AcMDB07 (Bylemans et al., 2018) showed the best performance in the freshwater river ecosystems in China (Zhang et al., 2020). Moreover, Blackman et al., (2021) found that the combination of two different 12S specific primer pairs resulted in a more complete detection of biodiversity of fish in a large tropical river network. Nevertheless, the efficiency of eDNA metabarcoding and primer pairs could differ across ecosystems, within species assemblages containing distinct lineages composition or complexity (Bellemain et al., 2010; Clarke et al., 2014), or depending on the physical properties of the system (Jo et al., 2019). Additionally, recent synthesis comparing multiple primer pairs have shown significant differences in performance using criteria combining primer specificity, taxonomic discrimination within the target clades, and in situ performance in diversity recovery (Collins et al., 2019; Zhang et al., 2020). In situ evaluation provides more direct information about the performance of primers under realistic conditions (Bylemans et al., 2018; Collins et al., 2019; Evans et al., 2016, 2017; Hänfling et al., 2016; Zhang et al., 2020). So far, studies performing comparisons of primer pairs in situ have mainly focused on sampling from one specific ecosystem, either marine or freshwater (Bylemans et al., 2018; Zhang et al., 2020).

The performance of primer pairs should be evaluated across multiple criteria (MacDonald & Sarre, 2017). Here, we propose a multicriteria comparative framework for assessing the performance of eDNA primer pairs, regarding (a) their capacity to bind and amplify a broad coverage of species using in silico PCR, (b) their capacity to discriminate among species, genera, and families, (c) their specificity and efficacy in situ across freshwater and marine environments as well as temperate and tropical environments. We applied this framework to two widely used primer pairs of the 12S mtDNA marker designed to amplify fishes: teleo (Valentini et al., 2016) and MiFish-U (Miya et al., 2015). The teleo primer pair targets a roughly 60 bp region located at the end of the 12S mtDNA strand because this area is the most variable across fish species (Figure 1; Thomsen et al., 2016; Valentini et al., 2016). The MiFish-U primer pair targets roughly 170 base pairs region at the beginning of the 12S mtDNA (Figure 1), which contains sufficient variability to identify fish to the species level (Miya et al., 2015).

In this study, we had the following objectives:

1. We compared the potential amplification performance of teleo and MiFish-U primer pairs across a broad range of mitogenomes of fishes including both Actinopterygii and Chondrichthyes. We expect that MiFish-U, optimized for Actinopterygii, should

recover more diversity within this taxon, while teleo should have a broader taxonomic range including Chondrichthyes.
2. We evaluated the species-level discrimination ability of teleo and MiFish-U by comparing the uniqueness of sequence patterns across species. Because the MiFish-U primer pair amplifies a longer DNA region, we expect it to show a better discrimination power at the species level.
3. We evaluated the ability of a neural network to assign sequences amplified by the teleo and MiFish-U primer pairs to genus and family levels based on patterns within the sequences. Because the MiFish-U primer pair amplifies a longer DNA region, we expect more phylogenetically conserved DNA patterns allowing a higher discriminatory power at the genus and family levels.
4. We evaluated the performance of teleo and MiFish-U primer pairs to specifically amplify DNA from fishes and recover species on eDNA samples in situ and whether this performance could vary across different ecosystems, from temperate to tropical and from marine to freshwater. We expect a higher performance of the teleo amplifying smaller fragments because DNA might be more degraded in tropical and marine environments.

2 | METHODS

2.1 | Primer sensitivity and species-specificity from in silico PCR

We applied in silico PCR to compare the MiFish-U (Miya et al., 2015) and the teleo (Valentini et al., 2016) primer pairs using USEARCH::search_pcr (v11.0.667_i86linux64). We used two different curated 12S rRNA reference data bases: the MIDORI database (Leray et al., 2018 GenBank release 240), which includes over 55 k metazoans and eukaryotic sequences, and the MitoFish data base (Iwasaki et al., 2013; Sato et al., 2018 release 362), which is a fish-specific database with over 4 k species. There is a partial taxonomic overlap between these two reference data bases. To ensure transparency and reproducibility, we did not alter them. In a first step, we screened the reference data bases for sequences covering both amplicons. This was essential to perform comparable in silico PCRs for both primer pairs. For the in silico PCR, we explored a variety of mismatches and allowed a maximum of three mismatches for each of the four primers individually. We ran multiple in silico PCRs for both primer pairs with different numbers of primer mismatches (0–3). Following Zhang et al., (2020), we also removed hits with mismatches at the last two positions of the 3' end.

Next, we analyzed whether unique taxa detected by both MiFish-U and teleo primer pairs presented a phylogenetic signal at genus levels. Because genus detection might be specific to selected primer pairs, we explored the phylogenetic signal of taxa by using the standardized effect size (SES) of the phylogenetic diversity index (PD, Faith, 1992) calculated with the R package PhyloMeasures (Tsirogiannis & Sandel, 2015). A negative SES PD below a value of −1.96 shows that the phylogenetic pattern in the variable is significantly clustered on the tree, whereas a positive value higher than 1.96 indicates a significant overdispersed signal. A value around zero means that the variable is randomly distributed on the tree. We used two distributions of 100 super-trees, for Actinopterygii (Rabosky et al., 2018) and Chondrichthyes (Stein et al., 2018) pruned at the genus level and corresponding to the MIDORI database. We calculated the mean and the standard deviation of the SES PD values for each primer pair at genus levels.

2.2 | Species specificity of amplified sequences by teleo and Mifish-U

We used the ensemble of amplified sequences from the MIDORI and MitoFish data bases to evaluate the species specificity of the teleo and MiFish-U primer pairs. Specifically, we computed the percentage of sequenced species per family for each primer pair as an indication of coverage for each data base, as well as two coefficients of taxonomic resolution from two different methods presented by Marquina et al., (2019): exclusive taxonomic resolution (BE) and taxonomic resolution (BS) per family. These approaches are originally designed within a sequence clustering framework when the diversity unit corresponds to clusters instead of individual sequences and can hence be used to define an appropriate barcoding gap. In the present work, we focus on individual sequences and do not consider sequence clustering. BE is the proportion of species whose amplified sequences are unambiguously identified. All sequences attributed to a species in the data bases must be unique to this species. If a species has two sequences, one unique and one shared with another species, it is considered not resolutive and not counted. We used a slightly different definition of the BE index from Marquina et al., (2019), where we allowed multiple sequences per species as long as those were not shared between different species. In the original approach, multiple sequences or clusters of sequences are not counted even if those belong to the same species. The coefficient represents the ratio of species considered resolutive/all amplified species for each family. For a ratio of 1, all species are resolutive, a ratio of 0.5, half species are resolutive within the family. BS corresponds to a less strict version of the BE index, where only one sequence unique to a species is necessary to be considered resolutive, as opposed to all sequences of a species for BE. If a species has two sequences, one unique and one shared with another species, it is still considered resolutive and is counted.

2.3 | Taxonomic predictability of amplified sequences by teleo and Mifish-U using neural networks

We used neural networks to evaluate the capacity of the teleo and MiFish-U primer pairs to amplify sequences that can be assigned to genus and family levels. This was performed using the sequences from the in silico PCR based on the recognition of k-mer patterns in

the sequences. For each of the MIDORI and MitoFish data bases, we generated four learning data sets: one for genus and one for species for both teleo and MiFish-U primer pairs. For the family data sets, we limited the selection to those with at least 10 genera in common between the teleo and MiFish-U assemblies. Similarly, for the genus data sets, we limited the selection of genera to those with at least 10 species in common. Considering the MIDORI data base, for the family sequences assignment, we selected 42 families containing 3033 sequences assigned to 2376 species for the MiFish-U data set, and 2820 sequences from 2373 species for the teleo data set. Similarly, for the genus assignment, we selected 54 common genera with 1124 sequences from 873 species for the MiFish-U data set, and 1065 sequences from 871 species for the teleo data set. The reverse complement for all sequences was added to the data sets for invariance with respect to the read direction of the sequences. We set aside 10% of the genera for each family and 10% of the species for each genus in all data sets to evaluate the generalization power of this approach over unseen taxa. We used those sequence data sets to train a set of neural networks per primer recognizing sequences at the genus and family levels each. We repeated the training of the neural network ten times, each time with holding 10% of the species and genera randomly. For each training repetition, we trained 10 neural networks per data set with four or five hidden layers, all of them either 32, 64, 128, 256, or 512 neurons wide. We use k-mer of length 5 since a previous study did not find differences between length from 3 to 6 (Nugent & Adamowic, 2020). We then created and trained a number of neural networks composed of four to five fully connected layers. The input layer is 1024 units wide representing the full 5-mer space of the canonical bases. The output layer is a softmax activated layer representing a probability distribution over the possible genus and family labels respectively. The hidden layers use the leaky Rectified Linear Unit (ReLU) activation function. We applied both dropout regularization to all hidden layers of the network and added a five percent base-flipping noise to all training inputs. We implemented our neural networks using the open-source software library TensorFlow v2.3 (www.tensorflow.org). Starting with randomly initialized parameters, we used a batch size of 1024 inputs and minimized over the sparse categorical cross entropy loss between the true and predicted labels. Then we used the trained model to predict the genus and family labels of the species that we did not observe in the networks before. We computed the summary performance by counting the mislabeled sequences at the genus and family levels.

2.4 | Primers evaluation using field-collected samples

We collected the eDNA in three different geographic regions consisting of different climatic zones (tropical and temperate) and different ecosystems (marine and freshwater). For each region, we collected two water filters in two different sites for a total of 12 samples/filters collected. The sampling sites included the Rhone River (France), in proximity of the cities of Jons and Brangues; the Mediterranean Sea around Carry-le-Rouet and the island of Porquerolles (France); and the Maroni River (French Guiana), upstream from the confluence with the Olemari river for the first site and downstream from the Yalou Patapte locality (ancient amerindian village) for the second site.

For the Rhône samples, DNA sampling was performed using a filtration device (SPYGEN VigiBOAT; www.spygen.com; nominal flow of 1.1 L/min^{-1}), a VigiDNA 0.45 μM cross flow filtration capsule (SPYGEN) and a disposable sterile tubing for each sample. For the Maroni samples, DNA sampling was performed using a filtration device (Vampire sampler), a VigiDNA 0.45 μM (SPYGEN), and a disposable sterile tubing for each sample. For Mediterranean Sea samples, the filtration device composed of an Athena® peristaltic pump (Proactive Environmental Products LLC; nominal flow of 1.0 L/min^{-1}), a VigiDNA® 0.2 μM cross flow filtration capsule (SPYGEN), and disposable sterile tubing for each filtration capsule. At each site, we performed two filtration replicates in parallel for 30 min, corresponding to about 30 L of water per filtration capsule. At the end of each phase of sampling, we emptied and then filled the filtration capsules with 80 ml of buffer solution (CL1; SPYGEN) and stored at room temperature. To prevent any type of contamination, we handled all materials with sterile gloves and cleaned frequently. We followed a strict contamination control protocol in both field and laboratory stages (Goldberg et al., 2016; Valentini et al., 2016).

Before the eDNA extraction begins, all people entering the laboratory first enter a sterile room where they get fully covered with a disposable laboratory suit, mask, laboratory shoes, overshoes, and gloves. The laboratory space is fully treated using 10% bleach and UV treatment. High air pressure and the constant regeneration of fresh air prevents contamination (Cilleros et al., 2019; Pont et al., 2018; Valentini et al., 2016). We performed the DNA extraction following the protocol described in Pont et al., 2018 for freshwater samples and in Polanco Fernandez et al., 2021 for marine samples. After the DNA extraction, we tested the samples for inhibition following the protocol described in Biggs et al. (2015) and no inhibitions were found, The amplification mixture contained 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems), 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl2, 0.2 mM each dNTP, 0.2 μg/μl bovine serum albumin (BSA, Roche Diagnostic), 0.2 μM of each primers (teleo or MiFish). We also added in the amplification mixture containing teleo primers, 4 μM human blocking primer (Civade et al., 2016). Twelve PCR replicates per sample were carried out. The primers were tagged on the 5' ends with unique nucleotide bases and with at least three differences between each tag (Pont et al., 2018; Valentini et al., 2016). Identical tags were assigned to both the forward and reverse primers in a given sample (i.e., filter), which allows for future identification of the respective samples (Thomsen et al., 2016; Valentini et al., 2016). The PCR mixture was denatured at 95°C for 10 min, followed by 50 cycles of 30 s at 95°C, 30 s at 55°C for teleo or 61.5°C for MiFish, and 1 min at 72°C, and a final elongation at 72°C for 7 min (Pont et al., 2018). One PCR negative control (also 12 replicates) per marker was sequenced in parallel. DNA extraction negative controls

were analyzed in Milhau et al., (2019) for River Rhône, in Cantera et al., (2019) for River Maroni and in Boulanger et al., (2021) for the Mediterranean Sea. As they were negative, we did not analyze them again for the present study. After amplification, the samples were titrated using capillary electrophoresis (QIAxcel; Qiagen GmbH) and purified using the MinElute PCR purification kit (Qiagen GmbH). Before sequencing, purified DNA was titrated again using capillary electrophoresis. The purified PCR products were pooled in equal volumes to achieve a theoretical sequencing depth of 500,000 reads per sample per marker. Two libraries, one for each marker, were prepared using the MetaFast protocol by Fasteris (https://www.fasteris.com/dna/?q=content/metafast-protocol-amplicon-metagenomic-analysis), which significantly limits the tag-jump problem (Taberlet et al., 2012). A paired-end sequencing (2 × 125 bp) was carried out using a MiSeq (2 × 125 bp) with the MiSeq Flow Cell Kit v3 (Illumina), following the manufacturer's instructions at Fasteris facilities.

Following sequencing, reads were processed to remove errors and analyzed using programs implemented in the OBITools package (http://metab arcoding.org/obitools, Boyer et al., 2016) as described in Valentini et al., (2016). The forward and reverse reads were assembled with the ILLUMINAPAIREDEND program, using a minimum score of 40 and retrieving only joined sequences. Then, we assigned the reads to each sample using the NGSFILTER software and we created a separate data set for each sample by splitting the original data set into several files using OBISPLIT. After this step, we analyzed each sample individually before merging the taxon list for the final ecological analysis. Strictly identical sequences were clustered together using OBIUNIQ. Sequences shorter than 20 bp, or with fewer than 10 occurrences were excluded using the OBIGREP program. The OBICLEAN program was then run. We discarded all sequences labelled "internal," which most likely correspond to PCR substitutions and indel errors. Taxonomic assignment of the remaining sequences was performed using the ECOTAG program with both a local reference database and a curated database of the sequences extracted from the release 138 (standard sequences). The curated database of the sequences extracted from the release 138 was built using the ECOPCR program (Bellemain et al., 2010; Ficetola et al., 2010). We built one database per marker and we selected only sequences present in both databases for the final curated data set. We used local databases for the Mediterranean Sea (Boulanger et al., 2021) and French Guiana (Cilleros et al., 2019) in which we retrieved only sequences containing both amplicons (Mifish-U and teleo). After the taxonomic assignment, only sequences with a similarity higher than 98% with the reference database were kept. After the filtering pipeline, the PCR negative controls were completely clean, and no sequence reads remained in those samples. We finally analyzed the specificity of MiFish-U and teleo as described in Collins et al., (2019). Immediately after demultiplexing and quality filtering (i.e., sequences longer than 20 bp, MOTU represented by more than 10 reads and MOTU), remaining reads are classified successively to Metazoa, Chordata, Aves, Mammalia, Actinopteri, Chondrichthyes, and Hyperoartia using a conservative 98% identity threshold in relation to the reference bases (EMBL for nonfish, and local databases for Actinopteri, Chondrichthyes, and Hyperoartia). Analyses were run in R (R Development Core Team, 2019).

3 | RESULTS

3.1 | Evaluation of primer sensitivity and species-specificity from in silico PCR

We compared the amplification performance of MiFish-U and teleo primer pairs using in silico PCR from the MIDORI and the MitoFish sequence data bases. We compared the in silico PCR amplifications of the MiFish-U and teleo primer pairs to the MIDORI and MitoFish sequence data bases. We report results for no and two mismatches, while other results can be found in Table 1. When considering no mismatch with the MIDORI data base, we found that teleo amplified a total of 60 orders, 279 families and 1171 genera, while MiFish-U amplified 23 orders, 59 families, and 118 genera. Similarly, with the MitoFish data base, teleo amplified a total of 59 orders, 271 families and 1216 genera, while MiFish-U amplified 22 orders 57 families and 126 genera. When considering two mismatches with the MIDORI database, teleo amplified 79 orders, 437 families, and 1829 genera, whereas MiFish-U amplified 79 orders, 394 families, and 1730 genera. With the MitoFish data base, teleo amplified 79 orders, 416 families, and 1900 genera, whereas MiFish-U amplified, 74 orders, 388 families, and 1792 genera. Considering three mismatches, the amplification of both primers further increased to offer a broad coverage (Table 1).

We found a difference in taxonomic breadth and specificity of amplification between both primer pairs investigated. In particular, considering two mismatches, teleo specifically amplified 4 orders and 37 families in the MIDORI data base, and 5 orders and 37 families with the MitoFish data base. In contrast, MiFish-U specifically amplified no orders and 8 families in the MIDORI data base and no orders and 9 families with the MitoFish data base (Figure S1). With both MIDORI and MitoFish data bases, teleo amplified a wider taxonomic spectrum not only within Actinopterygii but also Chondrichthyes and Petromyzontida. When accepting two mismatches, teleo was able to detect Myliobatiformes, Torpediniformes, and others shark orders (Selachii) as well as the phylogenetically very distinct group of lampreys (Petromyzontiformes). Considering the MIDORI data base and two mismatches for Actinopterygii and the unique genera detected by the MiFish primer pair (Figure 2), we found an average SES for phylogenetic diversity of −1.99 ± 0.18 across the 100 trees indicating that these genera are clustered (p = 0.013 ± 0.008). We found a similar result for the unique genera detected by the teleo primer pair for the same data base (SES PD = −3.1 ± 0.14; p = 0.00034 ± 0.0006) and, across the 100 trees supporting the result, that these genera are clustered. When focusing on the same data base and 2 mismatches for Chondrichthyes, we found that both the unique genera detected by MiFish-U (SES PD = −5.92 ± 0.56; p < 0.0001 and SD < 0.0001) and teleo (SES PD = −2.14 ± 0.53; p = 0.037 ± 0.04) are clustered on the phylogenetic tree. This indicates that both primer pairs had higher efficiency in some families compared to others. We

TABLE 1 In silico PCR results for a range (0–3) of primer site mismatches for both primer pairs with hits at order, family and genus level from the MIDORI and MitoFish data bases

		Mis-Match: 0			Mis-Match: 1			Mis-Match: 2			Mis-Match: 3		
		Total PCR Hits			Total PCR Hits			Total PCR Hits			Total PCR Hits		
		No 3'-MM PCR Hits			No 3'-MM PCR Hits			No 3'-MM PCR Hits			No 3'-MM PCR Hits		
		Order	Family	Genus	Order	Family	Genus	Order	Family	Genus	Order	Family	Genus
MIDORI	MiFish-U	248			4836			5759			6349		
		248 (−0)			4833 (−3)			5754 (−5)			6340 (−9)		
		23	59	118	65	331	1447	79	394	1730	79	432	1864
MIDORI	Teleo	4063			5750			6336			6381		
		4063 (−0)			5707 (−43)			6279 (−57)			6318 (−63)		
		60	279	1171	61	327	1443	79	437	1829	79	443	1844
MitoFish	MiFish-U	281			7039			8203			9382		
		281 (−0)			7036 (−3)			8197 (−6)			9371 (−11)		
		22	57	126	61	324	1490	74	388	1792	79	426	1936
MitoFish	Teleo	5793			8327			9370			9423		
		5793 (−0)			8280 (−47)			9307 (−63)			9353 (−70)		
		59	271	1216	67	359	1701	79	416	1900	79	422	1915

Note: The table provides the total number of PCR hits and the number of orders/families/genus detected by in silico PCR.

found similar results when considering the MitoFish data base and allowing two mismatches (Figure S2).

3.2 | Species specificity of amplified sequences by teleo and MiFish-U

We computed the coefficients of exclusive taxonomic resolution (BE) and taxonomic resolution (BS) for each family having (a) more than 100 species and (b) over 20 species per family represented in the data bases, which resulted in 46 families in MIDORI and 47 families in MitoFish. Within each of these data bases, we found that the species specificity largely varies among families. In particular, for the MIDORI data base, both teleo and MiFish-U primer pairs had lower species discrimination within a subset of the families (e.g., Acipenseridae, Cichlidae, Rajidae, Sebastidae, Salmonidae, Figure 3a,b). Overall, the longer fragments amplified by MiFish-U allowed a better level of species discrimination (mean BE = 0.805 ± 0.169, mean BS = 0.847 ± 0.147), than the shorter fragments amplified by teleo (mean BE = 0.687 ± 0.240, mean BS = 0.740 ± 0.222). We presented the results considering the MitoFish data base in (Figure S3a,b).

3.3 | Taxonomic predictability of amplified sequences by teleo and Mifish-U using neural networks

We found that both primer pairs produced sequences that can be assigned to the genus and family levels using neural networks (Figure 3c). With the exception of the smallest network (4 hidden Layers, 32 neurons wide), consistently underperforming, the results were comparable between the tested networks of different sizes. For the teleo primer pairs, the neural network predicted the genus correctly with ~96.5% accuracy and the family ~84.7% accuracy over all held out sequences. For the MiFish-U primer pair, we found a genus level accuracy of ~97.9% and a family level accuracy of 91.3%. While performances were high for both primer pairs, the neural networks trained on the MiFish-U data set consistently outperformed those trained on the teleo data set, especially at the family level. We presented the results considering the MitoFish database in (Figure S3c).

3.4 | In situ eDNA analyses across different ecosystems

To compare the two primer pairs in situ, we sampled eDNA in several marine and freshwater locations (Figure 4a,b). Across the two primer pairs, we recovered a total of 217 taxa identified to either the genus or the species level using teleo (26 orders, 65 families of Actinopterygii, 1 order and 1 family of Chondrichthyes and 1 order and 1 family of Petromyzontomorphi) and 183 taxa using MiFish-U (24 orders, 60 families of Actinopterygii). In the temperate freshwater environment of the Rhone River, using the teleo primer pairs, we found 13 orders and 20 families of Actinopterygii together with one order of the Petromyzontomorphi represented by the genus *Lamprea*, for a total of 53 taxa of which 36 were identified to the species level (Figure S4a). Using the MiFish-U primer pair, we also detected 14 orders (including Eupercaria) of Actinopterygii and 17

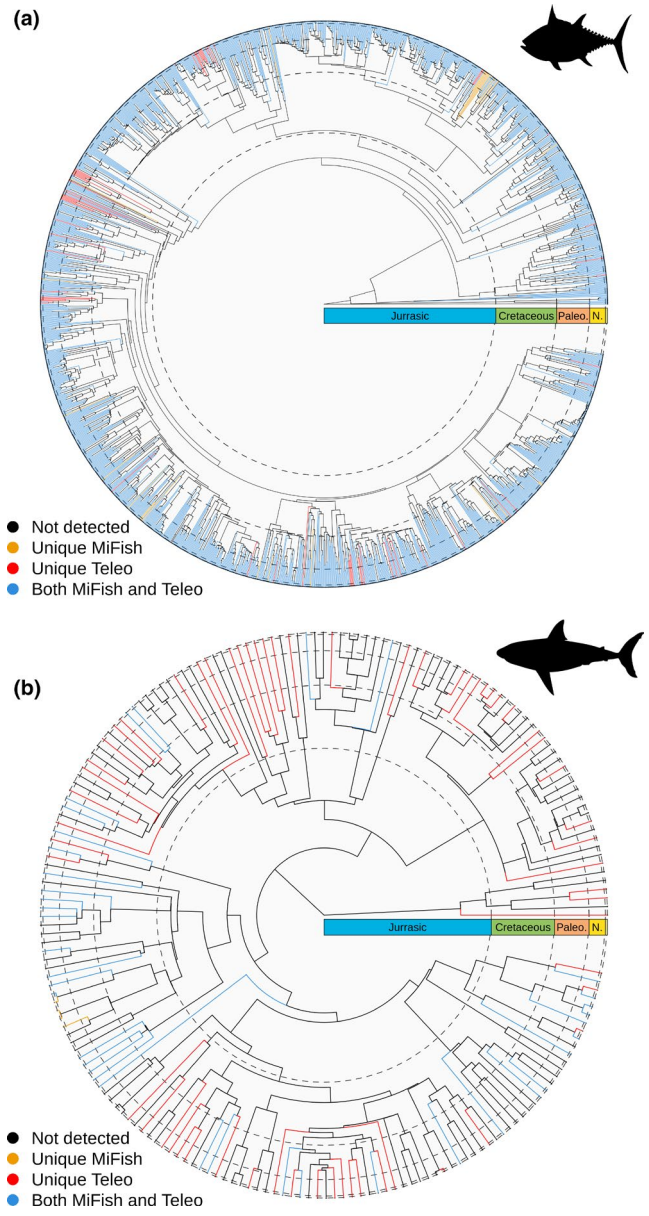

FIGURE 2 Phylogenetic distribution of the genera for Actinopterygii, (a) and Chondrichthyes (b), recovered uniquely by MiFish-U (in orange), uniquely by teleo (in red) and by both primer pairs (in blue) considering the MIDORI data base and allowing two mismatches. Families and genera not detected by either primer pairs are represented in black

different families for a total of 35 taxa of which 30 were identified to the species level (Figure S4a). Species detected by teleo and MiFish-U primer pairs did not completely overlap and we found that 15 species found with teleo were not detected with MiFish-U, while respectively 8 species found with MiFish-U were not detected with teleo. Combining the two primers pairs recovered a total of 46 taxa, more than each primer pair did alone. In the tropical freshwater environment of the Maroni River, using the teleo primer pair, we found 6 orders (including Eupercaria) and 29 families of Actinopterygii and one order of Chondrichthyes represented by the species *Potamotrygon orbignyi*, for a total of 121 taxa of which 102 were identified to the species level (Figure 4c, S4B). Using the MiFish-U primer pair, we detected 6 orders of Actinopterygii (including Eupercaria) and 29 different families for a total of 102 taxa of which we assigned 88 to the species level (Figure S4b). Species detected by teleo and MiFish-U did not completely overlap and we found that 27 species detected with teleo were not detected with MiFish-U, while respectively 14 species detected with MiFish-U were not detected with teleo. Combining the two primers pairs allowed recovering 141 taxa, more than each other did alone. In the marine ecosystem of the Mediterranean Sea, using the teleo primer pair, we found 13 orders and 18 families of Actinopterygii (including Eupercaria) and 1 order of Chondrichthyes, represented by the ray species *Myliobatis aquila*, for a total of 46 taxa of which 42 were identified to the species level (Figure 4C; Figure S4c). Using the MiFish-U primer pair, we detected 14 orders of Actinopterygii (including Eupercaria) and 19 different families for a total of 46 taxa of which we assigned 39 to the species level (Figure S4c). Species detected by teleo and MiFish-U did not completely overlap and we found that 12 species detected with teleo were not detected with MiFish-U, while respectively 10 species detected with MiFish-U were not detected with teleo. Combining the two primers pairs allowed recovering 57 taxa, more than each other did alone. The reference database was identical for both primers, implying that differences do not stem from database bias. We found that teleo and MiFish-U were highly specific with 99% of cleaned reads attributed to Actinopteri for both primer pairs (Figure S4e-g, Table S1).

4 | DISCUSSION

Given the critical importance of metabarcoding primers in eDNA analyses, a better understanding of primer properties and performances is central for an informed choice in biomonitoring studies (MacDonald & Sarre, 2017). Despite the increasingly wide application of eDNA metabarcoding in fish community surveys of diverse ecosystems (e.g. Cilleros et al., 2019; Polanco Fernandez et al., 2021; Valentini et al., 2016), studies comparing primer pairs remain generally limited in scope and do not consider multiple evaluation criteria (Collins et al., 2019; Zhang et al., 2020). Generally, appropriate primer pairs should (a) amplify most of the species within the target taxonomic group, (b) be able to differentiate at different taxonomic levels and (c) work in a variety of ecosystems (MacDonald & Sarre, 2017). The most recent broad analyses of multiple markers and primers showed a higher performance of longer 12S primer pairs (Zhang et al., 2020), but they are not the most used in fish eDNA studies. Our analysis focusing on the comparison of two broadly used primer pairs, teleo (Valentini et al., 2016) and MiFish-U (Miya et al., 2015), shows a high performance of both primer pairs, which fulfilled largely these criteria. In agreement with Bylemans et al., (2018a), we show that teleo is only marginally superior to MiFish-U both in silico and in situ, but combining both primer pairs showed the best performance in the three environments investigated. We suggest using

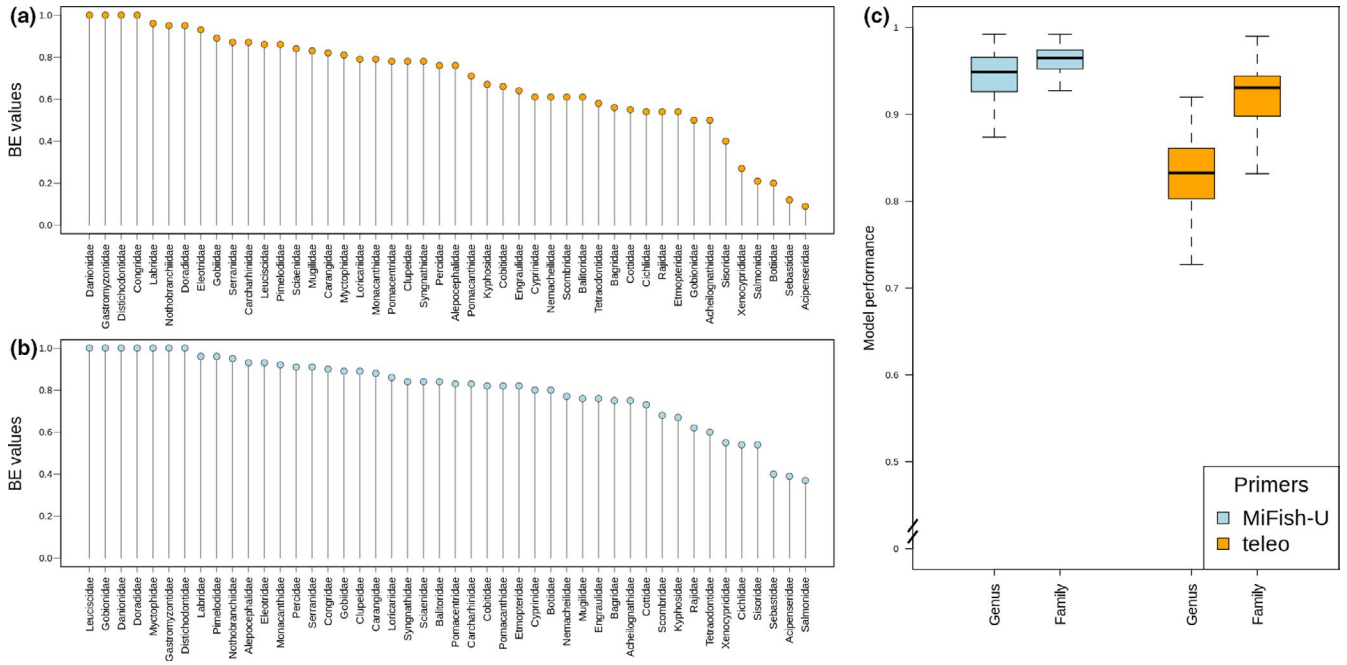

FIGURE 3 Species-specificity of (a) teleo and (b) MiFish-U across the different families. (c) Boxplot showing the performance of the neural network to predict the taxonomic assignment at the genus and at the family level for both MiFish-U and teleo for the MIDORI database

a combination of both primer pairs when possible, to detect fish assemblages more comprehensively.

Significant research efforts are still invested in the development and testing of primer pairs (Zhang et al., 2020), but we documented inherent trade-off in performance facets. In our analyses, while MiFish-U resulted in more specific taxonomic resolution, teleo was able to recover a larger number of species and a greater taxonomic range in our analyses. Teleo offers a general primer solution for fishes in both marine and freshwater ecosystems by covering a broad phylogenetic range (including Actinopterygii, Chondrichthyes, and Hyperoartia). The ability to amplify Actinopterygians and Chondrichthyes equally is a relevant feature when monitoring marine and tropical freshwater environments. The teleo primer pair can amplify in silico many Chondrichthyes, which is mirrored in our in situ analyses. For example, teleo was able to detect two ray species in both marine and freshwater environments and two Lamprey species from the Rhone River, while none of those species were recovered by MiFish-U. Although non-bony fishes represent a limited part of freshwater fish assemblages, detecting them has a particular interest for conservation as those species, such as the giant freshwater stingray (*Hymantura chaophraya*) in Asian rivers (Stone, 2007), are often threatened. Similarly, the sea lamprey (*Petromyzon marinus*) is an endangered species in European and North American rivers but is also an invasive species in the American great lakes where it is responsible for the decline in salmon production due to its parasitic feeding behavior (Hansen et al., 2016). Hence, our results support the finding in case studies, in which teleo was used. For example, Neotropical rivers host several species of strictly freshwater stingrays belonging to the potamotrygonidae family, but also several shark species that occasionally colonize freshwaters, which are recovered by eDNA analyses using teleo (Cilleros et al., 2019). Similarly, in marine environments, previous applications of the teleo primer pair have shown the ability to amplify and detect several shark species (Polanco Fernandez et al., 2021), showing that it may not be necessary to use two different primer pairs to detect those important species (Roff et al., 2016). The performance of teleo agrees with previous analyses (Bylemans et al., 2018b), but contrasts with a recent comparison across multiple primers developed for fishes (Zhang et al., 2020). An increasing number of studies reach a consensus that among barcode genes, the 12S mtDNA is highly effective to recover fish eDNA (Valentini et al., 2016). In particular, Zhang et al., (2020) found that the top six primer pairs that recovered the greatest numbers of fish taxa were all for the 12S region. However, in contrast with our results, Zhang et al., (2020) found a lower performance of teleo compared with other primers pairs including MiFish-U, both in silico and in situ. Zhang et al., (2020) used an uncurated version of the NCBI database during their in silico analyses, in which the coverage could have been higher for some portions of the 12S gene. In regard to their in situ analyses, PCR temperatures did not match the recommended PCR conditions for the use of teleo (Valentini et al., 2016), which could have led to suboptimal amplifications. Moreover, the resolution power of markers, where assemblages were dominated by the Cyprinidae family, poorly discriminated by teleo, could have led to a lower detection of taxa. In contrast to Zhang et al., (2020), our results show that teleo was performing slightly better in situ, but the shorter amplicon length from this primer pair was associated with a lower taxonomic discrimination ability.

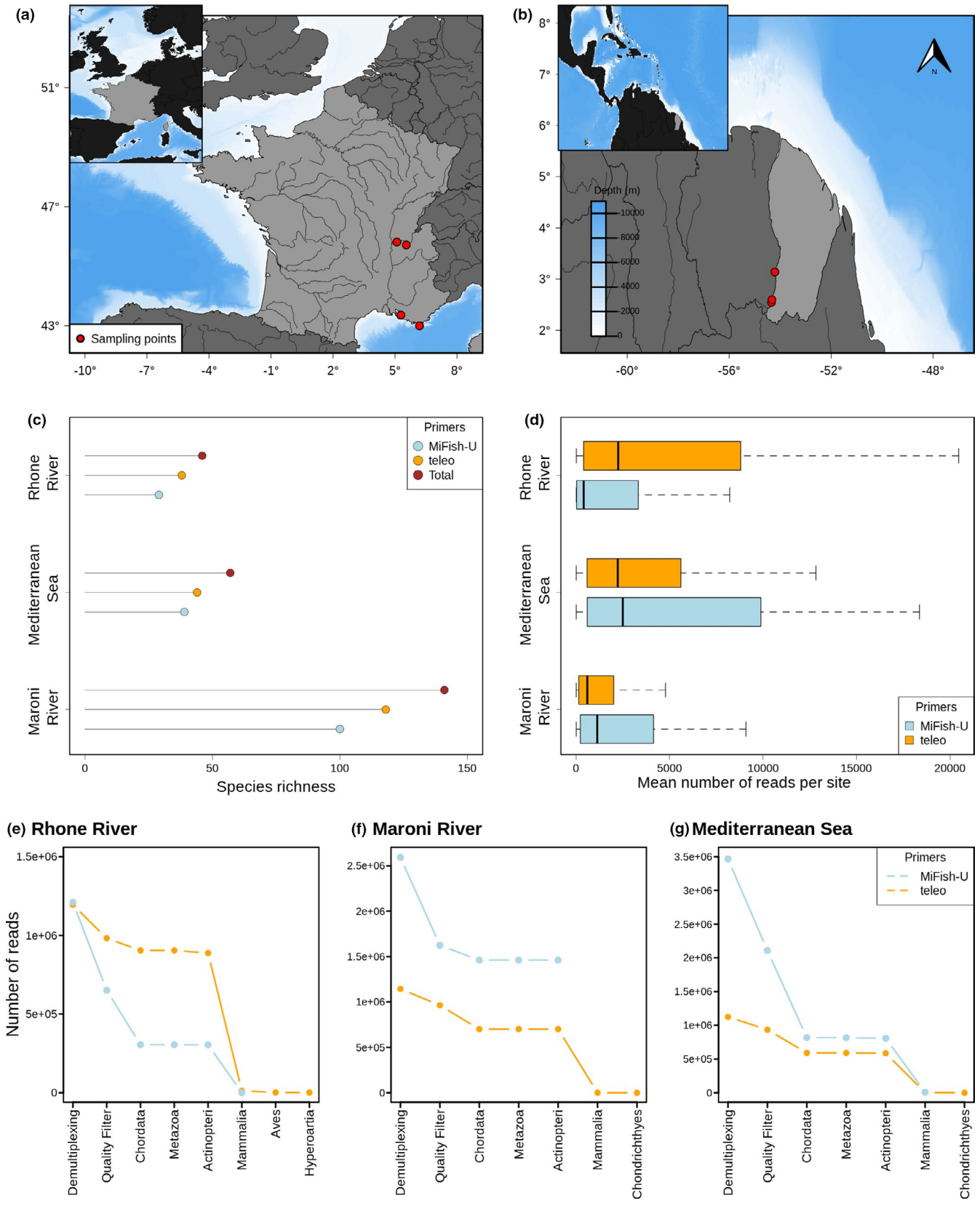

FIGURE 4 Sampling sites considering three different geographic regions in different climatic zones and different ecosystems. The sampling sites are the Rhone river (France; a), in proximity of the cities of Jons and Brangues, the Mediterranean Sea around Carry-le-Rouet and the island of Porquerolles (France; a); and the Maroni River (French Guiana; b), upstream from the confluence with the Olemari river for the first site and downstream from the Yalou Patapte locality for the second site. In situ evaluation of the species richness (c) and total number of reads (d) recovered from both MiFish-U and teleo primer pairs across three different ecosystems, the Rhone river, the Mediterranean Sea in temperate environments, and the Maroni River in tropical environments

Contrasted with teleo, MiFish-U was significantly more resolutive at the species level than teleo and showed a greater discrimination at higher taxonomic levels, which can be a useful feature in the absence of a complete reference database. MiFish-U showed a systematic superior discrimination both in the evaluation with the neural network and with the quantification of species-level specificity from in silico PCRs (Figure 3). As a consequence, MiFish-U could allow a better discriminative ability in environment with many phylogenetically closely related species, such as resulting from recent radiations (Doenz et al., 2018). Moreover, MiFish-U showed good performance in situ and recovered only marginally less species than teleo. This is corroborated by several empirical studies, where MiFish-U showed high detection performance, such as the assessment of marine protected areas (Gold et al., 2021) or in comparison with fish trawling surveys (Afzali et al., 2021), but MiFish-U recently showed limited performance in tropical freshwater environments (Jackman et al., 2021). In contrast with MiFish-U, teleo showed more limited power of taxonomic discrimination (Figure 3). In particular, species within fish families that experienced recent diversification were less discriminated such as Cichlidae (McGee et al., 2020), or Salmonidae (Crête-Lafrenière et al., 2012). We found that teleo had systematically lower species discrimination abilities than MiFish-U, which could be caused by a lower length of the amplified region containing less discriminatory motifs. Primer pairs amplifying short fragments could show better result in tropical environments dominated with rare species and assemblage with greater uniformity. Another advantage of primer pairs amplifying short fragments is that they can be "mass-produced" on, for example, Illumina NovaSeq (PE100 or PE150), while longer amplicons are restricted to, for example, Illumina MiSeq (PE250 or PE300) with a lower output. Hence, small fragments can be particularly relevant to get an estimation of biodiversity including less abundant species (Bylemans et al., 2018), but at the cost of reduced discrimination.

Despite the good performance of 12S primer pairs, a major current limitation is the low coverage of reference sequences (Collins et al., 2019): efficient recovery of biodiversity and accurate taxonomic assignments rely on the completeness and sequence quality of the corresponding databases (Bylemans et al., 2018; Elbrecht & Leese, 2017). When references are lacking, a complementary useful feature is the ability of DNA motifs on the sequences to be conserved phylogenetically, which would allow the eDNA reads to be assigned to higher taxonomic levels. We compared the taxonomic discrimination of MiFish-U and teleo, by training a neural network analysis to evaluate whether DNA patterns found in MiFish-U and teleo sequences allow deeper taxonomic discrimination at the genus and family levels. When we examined the individual predictions of the neural network along a fixed reference sequence, most confident, correct species-level predictions were achieved for most of those two 12S hypervariable regions. While the discrimination reached 97%, we show that overall, both primers had a reasonable capacity to predict the genus and a lower capacity to predict family taxonomic classes. Our results suggest that family assignments from incomplete reference databases can be uncertain, especially for smaller sequences. As these hypervariable regions are well-known and exploited features of 12S sequences, the ability to learn the importance of these regions from training data is a demonstration that the neural network captured important biological structure of 12S present in both MiFish-U and teleo sequences. There are two caveats to the application of neural networks to sequence taxonomic assignments. First, we have only examined positive identification over known families and genera, not how the results behave with regard to sequences belonging to unknown taxa. Second, we have done a limited exploration of network configuration and sizes only and a more exhaustive search may find better-suited network architectures yet. Despite those limitations, the neural network applied in our study provides a fair assessment of the family and genus assignment signal considered.

5 | CONCLUSIONS

We compared two commonly used primer pairs, MiFish-U and teleo and showed that each set displays strengths and weaknesses, but also a potential to be complementary. While teleo showed better amplification abilities in silico and better detection of taxa in situ, it displayed lower discrimination abilities at the species, genus, and family levels. In contrast, MiFish-U showed good discrimination abilities for most of the families investigated. MiFish-U and teleo only partially overlapped in simulated and empirical conditions. eDNA applications should aim toward a set of primers that offer the ability to obtain fish diversity in any aquatic ecosystems and the dual use of MiFish-U and teleo offers such as opportunity. We recommend that multiple primer pairs should be used in combination to increase species detection probability (Blackman et al., 2021; Evans et al., 2016; Miya et al., 2015; Shaw et al., 2016) when possible. Future design optimization of primers should not focus on unique primer pairs, but rather on primer combinations that allow for complete potential reads of species with eDNA. While teleo seems more efficient on its own, MiFish-U proposes already a set of four complementary primers that can be used to increase overall coverage (Kume et al., 2021). With the reduction of sequencing costs, an optimized, multiprimer approach should become a standard in future eDNA studies.

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CONFLICT OF INTEREST

AV and TD are working in a private company selling eDNA services. All authors declare that there is no conflict of interest regarding the publication of this article.

AUTHOR CONTRIBUTIONS

LP, and APF jointly designed this study, APF, AV, BF, CA, ER, JCW, and VM analyzed the data. LP, ER, and APF wrote a first draft and all the authors contributed to writing the manuscript.

DATA AVAILABILITY STATEMENT

The data set containing the raw eDNA data for the manuscript can be found in the following link: https://doi.org/10.6084/m9.figshare.14547078. Study Data for manuscript entitled "Comparing the performance of mitochondrial primers for fish environmental DNA across ecosystems". The full code for running the in silico PCR is provided in Genetic Diversity Centre (GDC)–Project Support Site (ethz.ch) https://www.gdc-docs.ethz.ch/Projects/p463/site/

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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