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SPECIAL ISSUE ARTICLE

Inferring functional diversity from environmental DNA metabarcoding

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Abstract

Environmental DNA (eDNA) metabarcoding is a reliable method to assess taxonomic diversity, but the incompleteness of genetic reference databases prevents the assignation of many sequences to a given species. Functional diversity (FD) is a key biodiversity facet to monitor, but it requires the identification of all species within communities to account for their trait values. So, the ability of eDNA-based inventories to estimate the "true" level of FD is unknown. Here, using fish surveys in two representative temperate and tropical rivers, with a quasi-exhaustive genetic and trait database, we measured the bias and variability of different FD indices when calculated with uncertainty in taxonomic assignment at the genus and family level. Our results show that when measuring FD indices with species randomly chosen within genera and families, the bias cannot exceed 30% from real observed FD values. The variability is higher for species-poorer communities and when those communities are composed of genera and families with high functional heterogeneity. Despite taxonomic uncertainty, our results demonstrate the potential of eDNA surveys to estimate reliable FD values.

KEYWORDS
eDNA metabarcoding, freshwater fishes, functional diversity, simulations, taxonomic uncertainty

1 | INTRODUCTION

Global pressure on biodiversity and negative consequences on ecosystems and human health have dramatically increased in space and magnitude in the last decade (Díaz et al., 2019; Jaureguiberry et al., 2022; Pecl et al., 2017). These changes could have irreversible consequences when ecosystem resilience reaches its point of no return (Mayor et al., 2013) or when ecosystem structure lacks adjustability to new conditions (Nagelkerken et al., 2020). A better monitoring of biodiversity states and trends is thus required to preserve ecosystem functioning and associated Nature's Contribution to People (Díaz et al., 2019). In this context, environmental DNA (eDNA) metabarcoding approaches provide new opportunities to detect a large breadth of taxa, even rare or elusive, and efficiently

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monitor biodiversity at unprecedented spatiotemporal scales (Deiner et al., 2021; Valentini et al., 2016). eDNA metabarcoding consists of retrieving DNA left by organisms in their surrounding environment, amplifying it using group-specific primers, and then detecting short molecular sequences (barcodes) that can be assigned to known taxa (Taberlet et al., 2012; Thomsen & Willerslev, 2015). However, only taxa that have been previously sequenced and referenced in genetic reference databases for the appropriate primers can be identified with this approach (Deiner et al., 2021; Marques, Milhau, et al., 2021).

While taxonomic diversity has long been used in ecological studies, it only represents one of the multiple biodiversity facets. Functional Diversity (FD), the diversity of traits in a given community, is the cornerstone between global change impacts and ecosystem functioning and has now become a key biodiversity metric to monitor (Carmona et al., 2021; Cinner et al., 2020; Su et al., 2021; Trindade-Santos et al., 2020). In an ideal situation, that is, with exhaustive species genetic and trait databases, so with markers able to discriminate all species in a given community, the computation of FD from eDNA metabarcoding is straightforward since it is based on species-level information. In practice, some, if not most, detected sequences remain unassigned to species as most genetic reference databases remain incomplete (e.g., Marques, Milhau, et al., 2021). These gaps explain why eDNA-based inventories are often composed of taxa assigned to species, genera, and families (e.g., Juhel et al., 2020). This taxonomic uncertainty may in turn challenge the estimation of FD, particularly in tropical species-rich areas that lack genetic reference data for most species (Marques, Milhau, et al., 2021). A solution to genetic database incompleteness is to exclude taxa assigned at higher ranks than species (or higher than genera) to avoid spurious trait variations (Aglieri et al., 2021). Nevertheless, ignoring these taxa can also bias FD estimates at the community level. To overcome this limitation, taxa only assigned at the genera or family level can be randomly chosen in the pool of species locally present within this genera or family and receive the associated traits (e.g., Marques, Castagné, et al., 2021). Indeed, we can hypothesize that species from the same genus or even the same family share similar traits (a pattern called *phylogenetic signal*, Losos, 2008) and hence play similar functions in ecosystems (Srivastava et al., 2012). This method has the advantage of considering more taxa but may create some uncertainty in FD estimates while remaining silent about the potential bias from the "true" FD based on an exhaustive assignment of all sequences to the species level. So, the extent to which FD of a given community can be accurately predicted from eDNA metabarcoding with sequences assigned to higher taxonomic levels is still unknown.

Here we propose to measure the bias generated by an incomplete genetic reference database (and thus taxonomic uncertainty) when assessing FD indices from eDNA taxa inventories. To this aim, we used data from two rivers with markedly distinct environmental and biodiversity characteristics, the Rhone and the Maroni rivers, as representatives of a European temperate and a Southern American tropical river, respectively. Both rivers benefit from almost exhaustive genetic and trait databases for fish. We simulated communities where species were randomly chosen within the same genera or family among the global regional fish fauna and then evaluated the extent to which this taxonomic uncertainty translates into biased and variable FD estimates from ground truth values. Finally, for each fish genus and family, we compared trait heterogeneity to the proportion of sequenced species in order to identify sequencing priorities towards more robust FD assessments from eDNA metabarcoding.

2 | MATERIAL & METHODS

2.1 | Environmental DNA inventories

Data used in this study were extracted from two surveys in Palearctic temperate Europe and in Neotropical South America. Europe inventories came from a 2017 survey on the Rhone River (Marques et al., 2020; Pont et al., 2018), and South America inventories came from inventories on the Maroni River in French Guiana carried out in 2017, where all the metadata associated are available on the French Guiana geoportal (geoguyane.fr) (Murienne et al., 2019).

For the Rhone River, the compilation of the 104 communities corresponding to sampling sites (Figure S1) comprises a total of 37 species, 34 genera, and 15 families. For the Maroni River, the compilation of the 37 communities (Figure S1) was composed of 120 species, 94 genera, and 39 families. There is no species, genus, or family in common between the temperate and tropical fish communities.

Environmental DNA was sampled using a VigiDNA 0.45 μm filtration capsule (SPYGEN) and a disposable sterile tubing for each sample. Each eDNA sample consisted in filtering 34 liters of water as recommended in Cantera et al. (2019). The input part of the tubing was placed a few centimeters below the surface in zones with high water flow. Sampling was achieved in turbulent area (rapid hydromorphologic unit) to ensure an optimal homogenization of the DNA throughout the water column. To avoid DNA contamination among sites, the operator always remained downstream from the filtration area and stayed on the bank (for streams) or on emerging rocks (for rivers). The capsule was then emptied and filled with 80 ml of CL1 conservation buffer (SPYGEN). After the DNA extraction, the amplification was carried out using "teleo" primers for the 12 S mitochondrial region (Valentini et al., 2016), then high-throughput sequencing was performed using a Illumina Hiseq 2500 for the Rhone River and a Miseq and a NextSeq for the Maroni River. Sequence reads were analyzed using the OBITools package (Boyer et al., 2016) following the protocol described in Pont et al. (2018). The ecotag function was then used for the taxonomic assignment of molecular operational taxonomic units (MOTUs) using a threshold of 98% of identity with the reference database. This step was done using the reference database updated from Cilleros et al. (2019) which references 255 Guianese fish species for Maroni samples and the database from Valentini et al. (2016) for Rhone samples. The MOTUs occurring with

a frequency bellow 0.001 per library sample were considered as tag-jumps and discarded (Schnell et al., 2015).

2.2 | Functional space and FD indices

To describe the main facets of fish ecology, we used 10 complementary morphological traits describing body parts involved in food acquisition and locomotion (relative maxillary length, oral gape position, body length, body elongation, eye vertical position, relative eye size, body lateral shape, pectoral fin vertical position, pectoral fin size, and caudal peduncle throttling) (Villéger et al., 2017). For measuring FD bias and variability linked to taxonomic uncertainty at the genus and family level, we chose all fish species from families potentially occurring in both Rhone and Maroni rivers (respectively 2808 and 3806 species). Trait values were extracted from the FISHMORPH global freshwater fish trait database (Brosse et al., 2021). For species that have missing traits, the matrix was completed using functional trait covariance (Goolsby et al., 2017). A principal component analysis (PCA) on the 10-dimension trait matrix was computed using the FactoMineR package (Lê et al., 2008; Figure S2). Seven axes explain more than 90% of the variance, so we used coordinates of species in this accurate functional space for computing FD indices (Mouillot et al., 2021).

Three FD metrics were selected because of their complementarity (Mouillot et al., 2013; Villéger et al., 2008). The Functional Richness (FRic) was computed as the volume of the convex hull shaping all the species in the functional space (Villéger et al., 2008). Functional Dispersion (FDis) was the average distance of species to their barycenter (Laliberté & Legendre, 2010). Functional Identity (FIde) was computed, for each axis, as the average species position (Mouillot et al., 2013). FD indices were calculated using functions of mFD package (Magneville et al., 2022). To assess functional heterogeneity among related species, we also computed FDis among all species within each genus and each family (Figures 1 and 2). All FD metrics were computed using presence–absence data, knowing that the number of reads can be strongly biased by the amplification efficiency that directly depends on the species (Piñol et al., 2015).

2.3 | Random communities to assess FD bias and variability

As FRic computation requires more species than axes, two communities from the Maroni and 10 communities from the Rhone were discarded because they host less than seven species. We thus considered 94 observed communities on the Rhone River with 8–28 species and 35 communities on the Maroni River with 8–89 species.

Our aim was to measure the bias and variability of FD indices (FIde, FRic, and FDis) while increasing uncertainty in taxonomic assignment. We therefore simulated assemblages with an increasing percentage of taxa assigned to a higher taxonomic level than species (genus or family). For example, if a community is composed of 10 species, the observed FD indices were computed on these 10 species without any taxonomic uncertainty (ground truth value). Then, we created 10 levels of taxonomic uncertainty (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%), using 1–10 taxa nonidentified at the species level. If nine taxa are identified at the species level and one is identified at the genus level, taxonomic uncertainty at genus level is 10%. In this case, estimated FD indices are calculated using nine taxa assigned to species and one species is randomly chosen among all species from its genus. For each level, we simulated 100 random communities (Figure 3). A total of 75,140 communities were thus simulated: 36,340 for the Maroni River (18,170 with uncertainty at the genus level and 18,170 with uncertainty at the family level) and 38,800 for the Rhone River (19,400 with uncertainty at the genus level and 19,400 with uncertainty at the family level). As a complementary analysis, we also tested an alternative procedure where the average value of functional traits from the genus (or family according to the taxonomic uncertainty level selected) was assigned to unknown species instead of a random sampling among functionally informed species from the same genera (or family). Results using random sampling and average functional values were similar so average value results are only presented in the Appendix S1 (Figures S4–S7).

We used Pearson correlations and linear regressions to assess the link between observed and estimated FD indices and measure both the bias (or deviation) and variability due to taxonomic uncertainty (Table S1). For FIde, the difference was scaled by the range of values on the corresponding axis of the PCA (Figure 6). Differences

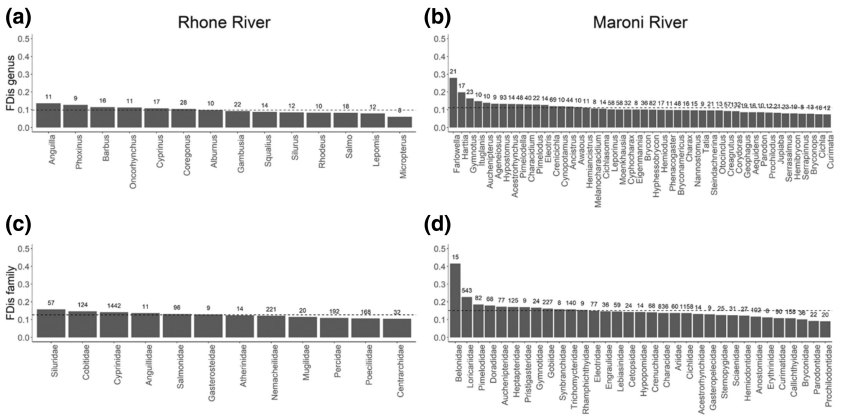

FIGURE 1 Functional heterogeneity (measured with FDis index) at the species level within genera (a, b) and families (c, d) present in Rhone (a–c) and Maroni (b–d) rivers. Numbers on top of each bar correspond to the number of species in the clade.

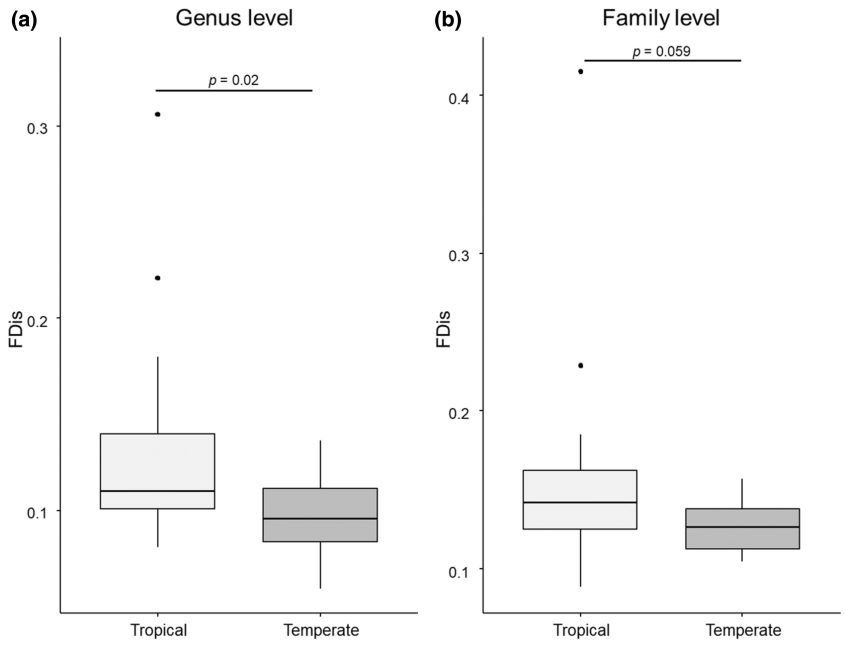

FIGURE 2 Comparison between functional heterogeneity (measured with FDis index) in tropical communities and temperate communities at the species level within genera (a) and families (b). FDis index was compared using a Wilcoxon test.

in FRic and FDis indices were not scaled because those indices have a range of 1 (Figures 4 and 5).

2.4 | Sequencing priorities

For all genera and families used in this study, we searched for a "teleo" sequence available in the public reference databases. For doing this, we extracted the sequences present in the release 142 extracted (standard sequences) from the ENA database and the fragments corresponding to the "teleo" marker were retrieved using "teleo" primer pairs and the ECOPCR program (Bellemain et al., 2010; Ficetola et al., 2010). The fish species amplified in silico were then compared to taxonomic fish lists available in FishBase (Froese & Pauly, 2021) to assess the percentage of species sequenced per genus or family. These results were then compared to the measured functional heterogeneity to set sequencing priorities per genus and family in each river.

3 | RESULTS

3.1 | Distribution of studied species in the trait space

Using the functional traits of each fish species, we plotted the position of all fish species on the two first axes of the multidimensional trait space. Species from the temperate river clustered in the center of the trait space, while tropical species were more widespread (Figure S2). Species from the *Anguilla* genus and from the Siluridae family presented maximum functional heterogeneity for temperate taxa (FDis of 0.12 and 0.15, respectively) (Figure 1a,c). The highest values of functional heterogeneity were observed for tropical genera and families, with a maximum for the *Farlowella* genus (FDis = 0.28) and for the Belonidae family (FDis = 0.41). Functional heterogeneity was significantly higher for tropical genera (mean FDis = 0.12) than for temperate ones (mean FDis = 0.098) (Wilcoxon test, $p < 0.05$, Figure 2).

3.2 | Observed and estimated FD values

For each FD index, linear regressions between observed and estimated FD values explained a similar amount of variation for taxonomic uncertainty at the genus and family level (Figure 3). Linear regressions explained between 80% of variation (for FIde on temperate communities) and 99% of variation (for FIde on tropical communities).

Bias in FRic values for temperate and tropical communities was significantly influenced by taxonomic uncertainty (Kruskal–Wallis test, $p < 0.05$, Figure 4a,b). Bias in FRic estimation was stronger for tropical communities when taxonomic uncertainty is increasing, and this trend was significantly stronger at the family than at the genus level (for the 80–100% taxonomic uncertainty class, the bias is 0.004% at the genus level and 0.006% at the family level) (Figure 4b,d and Figure S3). For tropical communities, bias in FDis was significantly higher at the family than at the genus level of uncertainty (Figure 5b,d and Figure S3). For some communities with uncertainty at family level, bias reached almost 10% (underestimation) from the observed communities (Figure 5c,d). Conversely, for temperate communities, the bias in FDis was significantly higher with taxonomic uncertainty at genus level than at family level (Figure 5a,c and Figure S3). However, variability was wider at the family than at the genus level. Bias in FIde on the first dimension was significantly higher at the family than at the genus level for tropical communities (Figure 6b,d and Figure S3). The maximum bias between observed and estimated communities reached 30% for this index. The

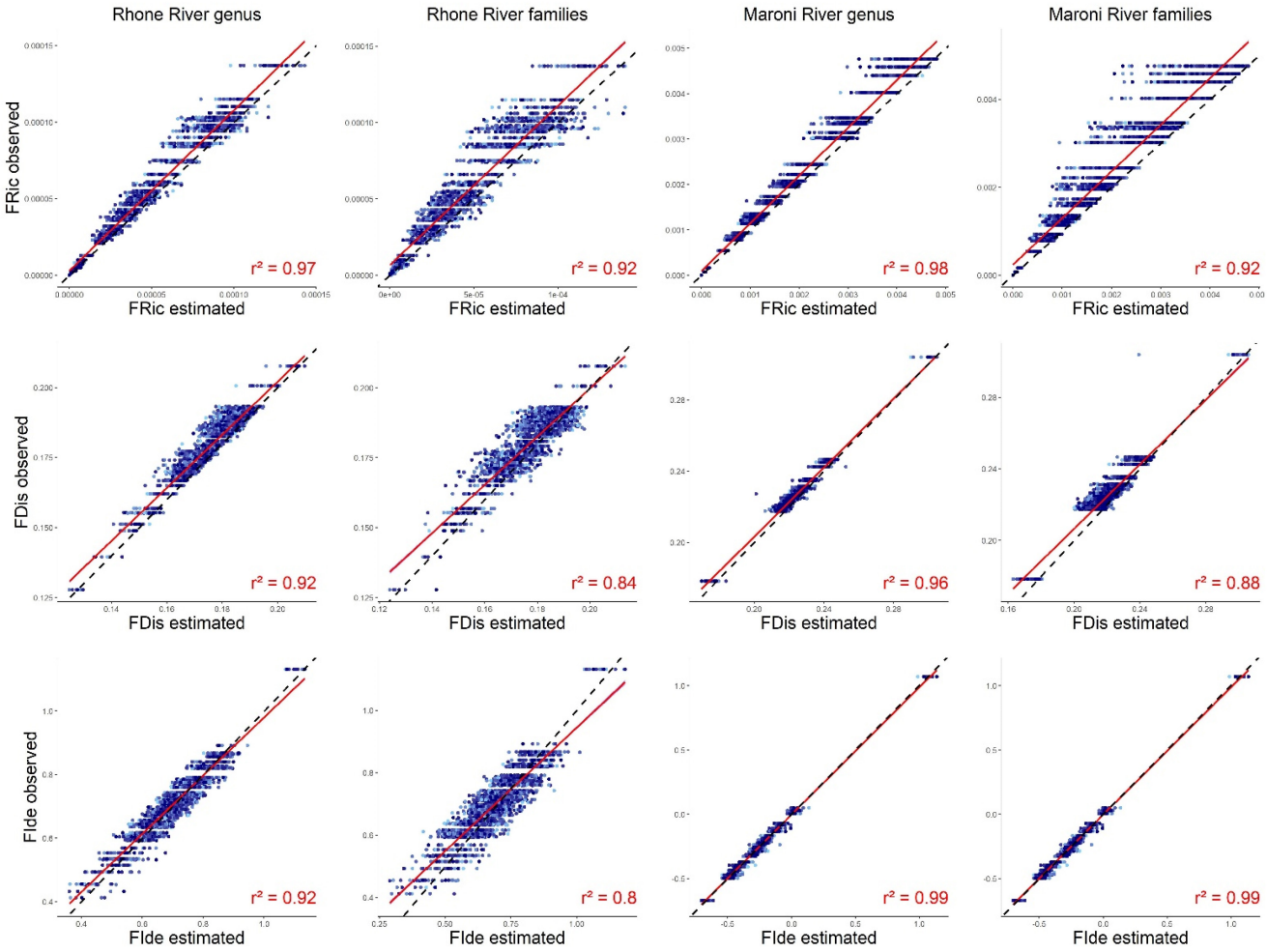

FIGURE 3 Correlation of the three functional diversity indices between observed and simulated communities for tropical and temperate rivers at the genus and family uncertainty levels varying from 0 to 20%. The black dot line represents the $y = x$, the red line represents the linear regression, and the shades of blue represent the size of communities (light to dark shades indicate an increase in species richness).

FIde mean variation for temperate communities was almost null, with a higher variability at the family level (Figure 6d).

3.3 | Functional heterogeneity highlights sequencing priorities

We identified genera and families composed of species with high functional heterogeneity and low proportion of sequenced species (Figure 7). For temperate communities, the *Barbus* and *Cyprinus* genera but also the Siluridae and Cobitidae families were the least sequenced and the most functionally diverse taxa. For tropical communities, the *Gymnotus* and *Characidium* genera and the Belonidae and Trichomycteridae families were identified as sequencing priorities.

4 | DISCUSSION

At global scale, species and functional diversity of fishes is higher in the tropics (McLean et al., 2021; Rabosky et al., 2018; Toussaint et al., 2016). This trend is also true for genera and families belonging to the two studied rivers, with intragenus and intrafamily functional heterogeneity being higher for tropical freshwater fishes than for temperate ones (Figures 1 and 2). Tropical species occupy many ecological niches under various environmental conditions (Albert et al., 2020), and when species from the same genus (or family) live in different ecosystems, a high functional heterogeneity is observed (Mori et al., 2019). Contrarily, temperate species are less disparate and have a lower breadth of functions within genera and families (Figure 1 and Figure S2). This was due to the last quaternary glaciation that caused massive fish extinctions in western European rivers and shaped a narrower breadth of functional traits than in tropical areas (Reyjol et al., 2006).

Comparing the FD indices between observed and simulated communities across a wide range of species uncertainty at both genus and family levels revealed a low functional variability. Indeed, functional variability ranged from 0 to 30% according to the FD indices considered, even for communities entirely assembled randomly at the family level, suggesting that taxonomic uncertainty has little influence on FD estimates for fish communities in both temperate

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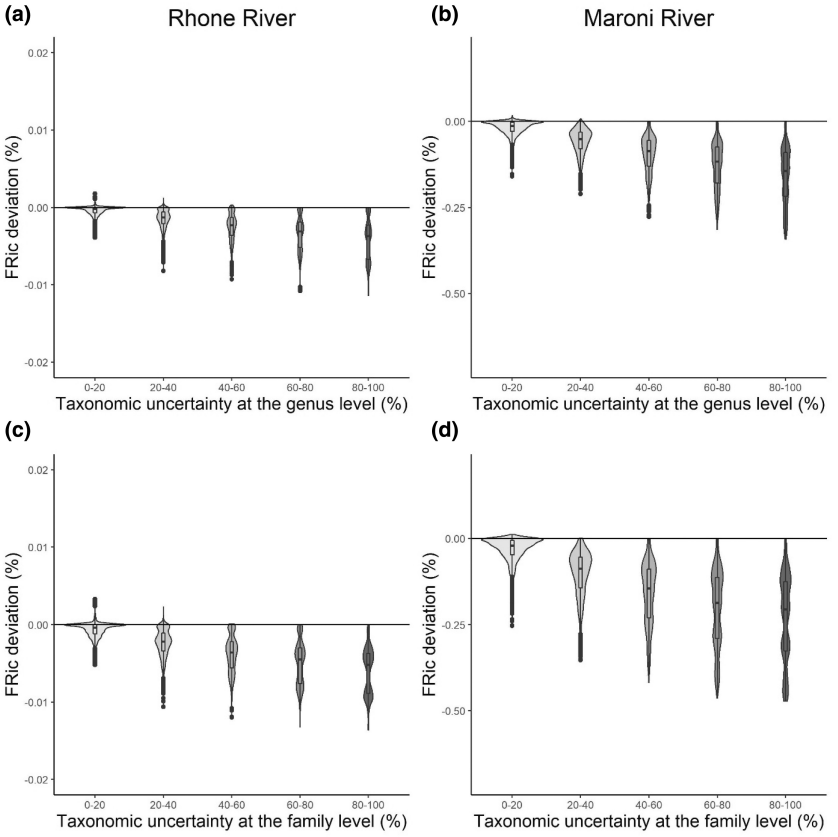

FIGURE 4 Bias in the FRic index between observed and simulated fish communities for the Rhone (a, c) and Maroni (b, d) rivers. The bias is expressed in percentage and the taxonomic uncertainty is expressed in percentage of assignation at the genus (a, b) or family (c, d) level. Light to dark shades indicate an increase in taxonomic uncertainty.

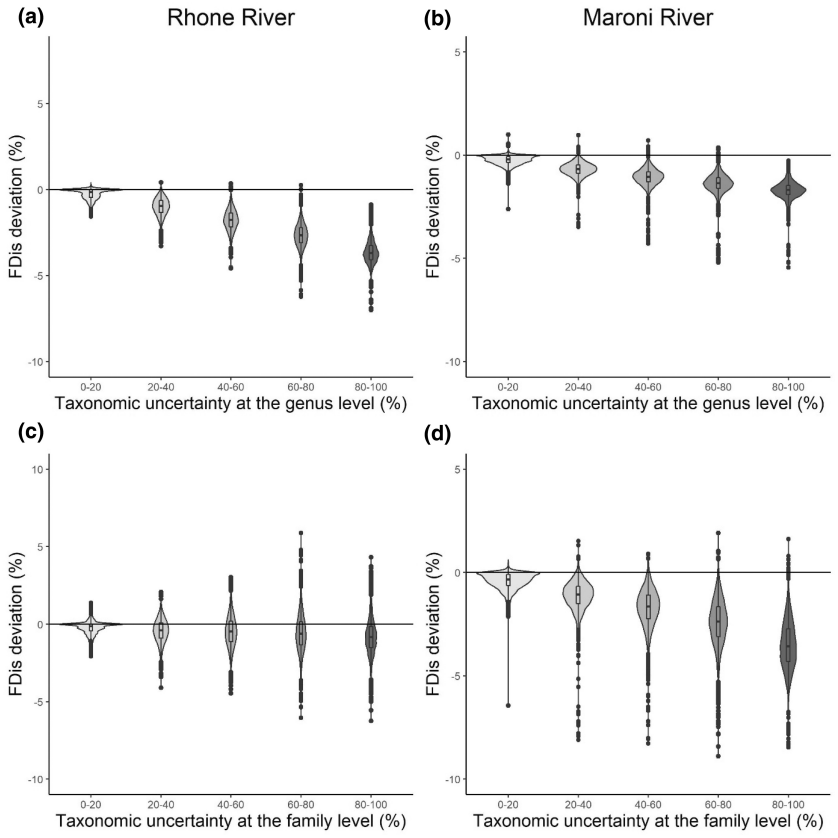

FIGURE 5 Bias in the FDis index between observed and simulated fish communities for the Rhone (a, c) and Maroni (b, d) rivers. The bias is expressed in percentage and the taxonomic uncertainty is expressed in percentage of assignation at the genus (a, b) or family (c, d) level. Light to dark shades indicate an increase in taxonomic uncertainty.

and tropical rivers. Bias was nevertheless higher with taxonomic uncertainty at the family than at the genus level. Considering uncertainty at a higher taxonomic level implies a random assignation of species from a larger species pool, which therefore increases potential functional heterogeneity. Indeed, species among a family are more distantly related than among a genus, and thus, under

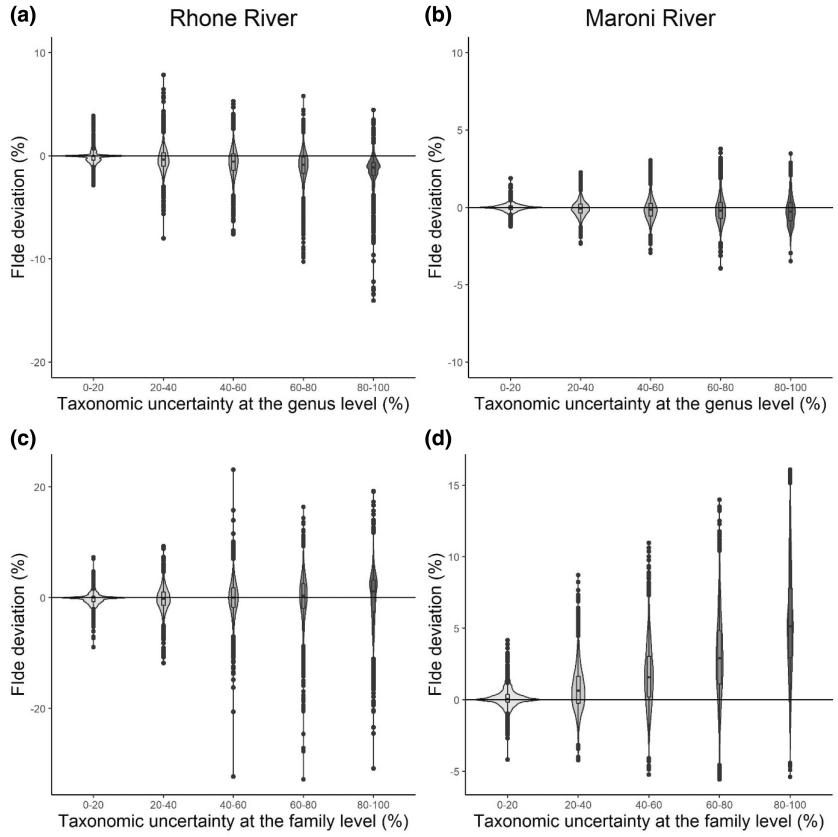

FIGURE 6 Bias in the FIde index between observed and simulated fish communities for the Rhone (a, c) and Maroni (b, d) rivers. The bias is expressed in percentage and the taxonomic uncertainty is expressed in percentage of assignation at the genus (a, b) or family (c, d) level. Light to dark shades indicate an increase in taxonomic uncertainty.

the hypothesis of phylogenetic signal in species functional traits (Srivastava et al., 2012), species functional characteristics should be more heterogeneous among families than among genera. Moreover, because of the functional heterogeneity of some genera and families (Figure 1), some biases are relatively high, leading to values of FD indices for simulated communities that markedly differ from those measured on real observed communities (underestimation of 30%, see Figure 6). To reduce this deviation, we suggest focusing future sequencing efforts on species belonging to genera and families with high functional heterogeneity. Here we show that for both temperate and tropical communities, the species for which sequencing is a priority belong to some species-rich taxa with low interest as food or recreational resources for humans (e.g., Cobitidae or Trichomycteridae families), leading to a lower sequencing effort than for taxa more related to human uses (Marques, Milhau, et al., 2021). In the same way, taxa with unusual morphologies and extreme functional traits (e.g., Belonidae family, or *Farlowella* genus; Su et al., 2019) are also pointed out as sequencing priorities. These taxa not only share extreme traits but also a wide variation of traits among species (Su et al., 2019), explaining why sequencing should be directed in priority on most, if not all, those species. It is a prerequisite to reduce functional uncertainty in eDNA-based studies (Marques, Castagné, et al., 2021).

Among the functional indices tested, FRic is the least affected by taxonomic uncertainty resulting from incomplete reference databases. Even for simulated communities exclusively made of random species sorting at the genus or family level, bias from observed communities never exceeds 1% in both temperate and tropical communities (Figure 4). This bias is slightly more important for tropical communities, mainly because tropical genera and families are functionally more heterogeneous than temperate ones. The bias in FDis is also higher for tropical communities for the same reason (Figure 5). The highest bias (almost 30% underestimation) observed in this study are for the FIde index, particularly for temperate communities (Figure 6). Functional heterogeneity is higher for tropical clades (Su et al., 2019), increasing therefore FIde variability (Figure 2). This trend is nevertheless balanced by the higher species richness in tropical communities compared to temperate ones (Oberdorff et al., 2011), which probably buffers FIde variability, explaining therefore the low FIde bias for tropical rich communities even when taxonomic uncertainty is high.

Because of the gaps in reference databases, eDNA-based inventories can generate communities made of taxa at the species, genus, or family level, and this uncertainty can lead to biased FD indices. Here we show that bias in FD estimations from eDNA data is due to functional heterogeneity within genera or families. This increases the probability to randomly consider species with distinct traits from those which are really present in the community but not detected at the species level because of gaps in genetic databases. Since FD is an essential component of ecosystem functioning (van der Plas, 2019) and a key indicator of human pressure (Cinner et al., 2020; Trindade-Santos et al., 2020), the ability of eDNA-based inventories to infer FD needs a deep evaluation. We unambiguously show, at least on fish communities from temperate and tropical rivers, that taxonomic

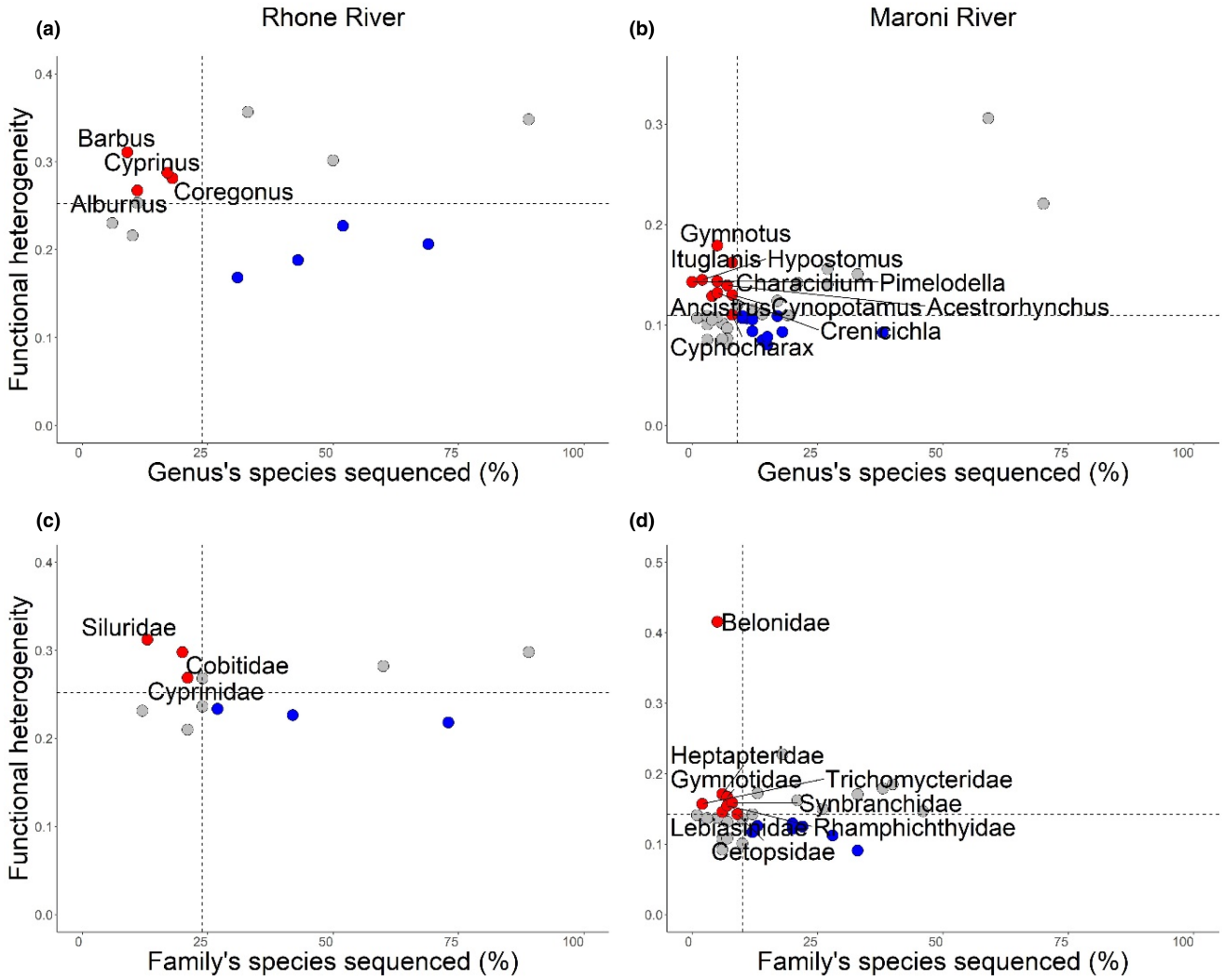

FIGURE 7 Relationships between species functional heterogeneity and the percentage of species sequenced within genera and families in the Rhone (a–c) and Maroni rivers (b–d). Top sequencing priority genera and families are those with high functional heterogeneity (FDis) and low percentage of sequenced species for the 12S primer like *Barbus* or Siluridae (red). Low (blue) and medium (gray) priority genera and families are those with low FDis and high percentage of sequenced species for the 12S primer and are not labeled. Dotted lines represent the median for each axis.

uncertainty inherent to eDNA surveys given the incompleteness of genetic reference databases is not a limiting factor to provide robust FD estimates. Since eDNA inventories capture more taxonomic diversity than classical surveys, at least for fish (Mathon et al., 2021; Milan et al., 2020; Polanco et al., 2021), we can conclude that despite taxonomic uncertainty, FD estimates obtained with eDNA are more robust and more exhaustive than those obtained with classical surveys (Marques, Milhau, et al., 2021), making eDNA an appropriate tool to monitor FD. Focusing the future sequencing effort on taxa less sequenced and highly functionally heterogeneous (Figure 7) will help to estimate FD indices even more confidently when using eDNA inventories. This sequencing strategy based on functional heterogeneity within genera and families present in the Rhone and Maroni rivers could be thus generalized to the world fish fauna to make eDNA approaches efficient to measure fish functional diversity across the world freshwater ecosystems that are recognized among the most threatened while supporting irreplaceable ecosystem services (Albert et al., 2021).

AUTHOR CONTRIBUTIONS

TM, JM, TD, and DM conceived the ideas and designed methodology; CC, TM, SB, SV, AV, and DM analyzed the data. Manuscript revision was done by CC. All authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

TM, AV, and TD are research scientists at a private company specializing in the use of eDNA for species detection.

DATA AVAILABILITY STATEMENT

Metadata can be found for the Rhone River in the paper of Pont et al. (2018) and for the Maroni River in the paper of Murienne et al. (2019) or on the French Guiana geoportal (geoguyane.fr). The Illumina raw sequence data used in this study are available under accession code https://doi.org/10.5061/dryad. t4n42rr for Rhone samples and https://doi.org/10.5061/dryad.pvmcvdnmr for Maroni samples.

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SUPPORTING INFORMATION

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