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Abstract

Freshwater ecosystems are experiencing one of the highest rates of biodiversity decline among extant ecosystems. To inform effective conservation actions, it is imperative to develop reliable monitoring techniques to assess the species richness of freshwater communities. In this study, we applied for the first time an eDNA metabarcoding assay on six watercourses in Liguria, northwestern Italy. Our first aim was to validate this method as a reliable monitoring tool for Ligurian fish communities. To reach this goal, we compared the results of the eDNA-based sampling with those obtained from two electrofishing campaigns carried out during the same season. The eDNA-based approach yielded congruent results with electrofishing data and showed a slightly higher resolution since it was able to detect two threatened species that were not detected by traditional monitoring. Just one species that eluded eDNA detection was electrofished instead. Thanks to a multi-marker metabarcoding approach we were also able to detect other vertebrate species living in, or associated with, the sampled watercourses, such as aquatic birds and amphibians. Overall, our results confirmed that aquatic eDNA assay proves to be a valuable tool to monitor freshwater-related systems and to inform efficient management and protection schemes for such habitats.

Keywords

eDNA, biodiversity monitoring, 12S mtDNA region, teleosts, Tele02, Vert01

Introduction

Biodiversity loss is a major threat to the survival of various ecosystems and, ultimately, to human well-being, through its negative impact on the provision of ecosystems' goods and services to human societies (Ceballos et al., 2015). Freshwater environments are among the most species-rich ecosystems on the planet and are characterized by the presence of many endemic species. Population trend data, however, show that these environments are experiencing rapid loss of biodiversity and place them among the most threatened extant habitats (Evans et al., 2016; Dudgeon, 2019; Hughes, 2021; Williams-Subiza and Epele, 2021). The observed decline in freshwater species is due to many threats that can be classified in six categories: flow regulation, pollution, land-use change, overexploitation, invasive species and global climate change (Lacoursière-Roussel et al., 2016; Dudgeon, 2019; Su et al., 2021). It has recently been demonstrated, however, that appropriate conservation actions and protection policies are successful to counteract such threats and can prevent biodiversity loss (Langhammer et al., 2024).

A prerequisite for the development of protection policies of habitats and native species is to perform reliable monitoring surveys, which allow the assessment of the species richness within an ecosystem and the conservation status of the different species (Aglieri et al., 2021; Seymour et al., 2021). Traditional methods for biodiversity monitoring are based on visual observation or capture-based samplings, which are both time consuming and have recognised limitations such as identification errors, human disturbance on the habitat and other difficulties of application (Lacoursière-Roussel et al., 2016; Deiner et al., 2017). In recent years, environmental DNA (eDNA) analysis has been confirmed as an innovative biodiversity monitoring tool and has been widely used, with a special focus on aquatic environments (Hänfling et al., 2016; Shaw et al., 2016; Seymour et al., 2021; Schenekar, 2023). The eDNA approach is more sensitive than the above-mentioned traditional methods, since it allows early detection of rare and invasive species, as well as juvenile and cryptic stages of organisms (Evans et al., 2017; Thomsen & Willerslev, 2015; Ballini et al., 2024). It also proved to be a non-invasive and cost-effective method (Evans et al., 2017; Taberlet et al., 2018; Pawlowski et al., 2020). This approach has been applied in monitoring programmes serving various purposes, such as species identification, species richness estimation, community monitoring and development of biotic indexes (Ruppert et al., 2019).

Several studies have shown the effectiveness of eDNA techniques in characterising the fish communities of freshwater environments, and in assessing spatial and temporal changes in the composition and richness of such communities (Thomsen et al., 2012; Thomsen & Willerslev, 2015; Djurhuus et al., 2020; Seymour et al., 2021; Jeunen et al., 2022). Such studies confirmed that the fish community composition derived from eDNA metabarcoding is comparable to - and it often even outperforms - the data gathered using traditional methods (Civade et al., 2016; Hänfling et al., 2016; Shaw et al., 2016; Czeglédi et al., 2021; McColl-Gausden et al., 2021; Penaluna et al., 2021; Golpour et al., 2022). In this context, the completeness and accuracy of DNA barcode reference libraries is still one of the main challenges of the

metabarcoding approach (Weigand et al., 2019), mainly because gaps in sequence coverage vary considerably among taxonomic groups, geographic regions, and genetic markers (Tzafesta et al., 2022; Claver et al., 2023). The discriminatory power of short barcode regions may also prevent the identification of congeneric, sister and highly introgressed species (Jackman et al., 2021; Claver et al., 2023; Wang et al., 2023). A multi-marker metabarcoding approach can improve species resolution, increase species detection and provide a more comprehensive assessment of freshwater-related communities (Robinson et al., 2022).

In this study, we applied for the first time an eDNA metabarcoding assay on six watercourses of Liguria (northwestern Italy), a region known for its high rate of endemism and the presence of numerous protected species (ISPRA, 2023). Our aim was twofold. First, we compared the results obtained using our eDNA metabarcoding approach with data obtained from electrofishing aimed to assess the fish biodiversity of the target rives and carried out during the same season. We then tested the efficiency of aquatic eDNA metabarcoding analysis in detecting stream-associated vertebrates, such as aquatic birds and amphibians. To achieve our goals, we used a multi-marker metabarcoding approach by sequencing two hypervariable fragments of the mitochondrial 12S rRNA gene commonly used for vertebrates and fish identification (Vert01 and Tele02: Riaz et al., 2011; Taberlet et al., 2018).

Material and Methods

Study area

The sampling was performed at six rivers in western Liguria (northwestern Italy): Roia, Bevera, Argentina, Carpasina, Nervia and Tanaro (Fig. 1). The Tanaro basin is located at the border of the Padano-Venetian ichthyogeographic district, while the other watercourses flow into the Ligurian Sea. Parts of the Roia and Bevera rivers are located in France (Bianco, 1990; 1995). Based on long-term traditional monitoring programs and historical data, approximately 12 fish species occur in these drainage basins, most of which belong to the families Leuciscidae (*sensu* Schönhuth et al., 2018), Cyprinidae and Salmonidae (Table S1) (Borroni, 2004; Borroni, 2005; Ciuffardi, 2006; Ciuffardi et al., 2015). The area is home to several endemic and threatened vertebrate species according to the IUCN, such as the critically endangered European eel, the data deficient freshwater blenny, the near threatened dunnock and the vulnerable common toad.

Electrofishing

Electrofishing surveys were conducted in March and May 2022 by using a backpack electrofisher (model ELT 60 II GI). The sampling was carried out in direct current (DC) to minimize trauma to the fauna. Four watercourses were monitored: Roia, Bevera, Carpasina, and Tanaro. The Bevera was sampled at two different locations (Olivetta San Michele and Torri), while for the other rivers only one site was selected.

Sampling covered all morphological units found in the surveyed sections, according to the MesoHABSIM methodology (Parasiewicz et al. 2013; Vezza et al. 2017).

eDNA sampling

Environmental DNA sampling was carried out in October 2022 at the six selected rivers. One sampling point per watercourse was selected, considering the electrofishing surveys. Sampling was performed following procedures described in Ballini et al. (2024). In brief, for each river the water samples were collected on both banks of the river and midstream at six randomly selected spatial replicates along a 150 m-long transect. Each replicate consisted of one litre of water collected in a plastic jar previously sterilised with sodium hypochlorite. Water samples were filtered on-site using a portable handheld vacuum pump connected to a polypropylene flask (Thermo Fisher Scientific Inc.). Sterile disposable filter units and nitrocellulose membranes with a pore size of 0.2 μm (ThermoFisher Scientific Inc.) were used. All filters were preserved in absolute ethanol immediately after collection, transported to the laboratories of the Biology Department of the University of Florence and stored at -25°C until DNA extraction. At each site, a jar containing one litre of DNA-free deionised sterile water was left open for two minutes. The water from such jars was then filtered on-site and used as field negative control.

DNA extraction, library amplification and sequencing

In total, we analysed 44 samples, 36 of which were water samples, six field blanks, one PCR negative control and one PCR positive control. Environmental DNA purification, Polymerase Chain Reaction (PCR) amplification and library preparation were performed in a laminar flow cabinet using sterile equipment to prevent exogenous DNA contamination. Strict protocols for sterilisation and contamination control were used for all molecular work.

Environmental DNA was extracted from membrane filters using the ZymoBIOMICS™ DNA Miniprep Kit according to the manufacturer's instructions. DNAs were eluted in a final volume of 50 μL of sterilised water and then stored at -25 °C. After the purification, the DNA extracted from each sample was quantified using the Invitrogen™ Qubit™ 4 Fluorometer and the Invitrogen™ Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific Inc.).

For eDNA amplification, we used the primer sets Tele02 and Vert01 (Riaz et al., 2011; Taberlet et al., 2018), targeting two different fragments of the mitochondrial 12S rRNA of ~167-bp and ~97-bp respectively.

Amplicon libraries were prepared following the dual-PCR protocol for Illumina platforms (Bourlat et al., 2016). In the first PCR step, barcode markers were amplified in a total volume of 25 μL using 1X Invitrogen™ Taq DNA Polymerase PCR Buffer, 1 U of Taq DNA Polymerase (Thermo Fisher Scientific Inc.), 2 mM $MgCl_2$, 0.3 mM dNTPs, 0.5 μM of each primer enriched with an Illumina adapter overhang (Table S2) and 1 μL of template DNA. The following amplification conditions were used: 5 min at 94°C, 35 cycles of 94°C for 30 sec, 54°C (Tele02) or 49°C (Vert01) for 30 sec, 72°C for 1 min, then 10 min at 72°C. For each sampling site, the amplification of eDNA samples was performed on three technical replicates. Negative field controls, an amplification negative control (containing DNA-free ddH_2O instead of template DNA) and an amplification positive control were included in the same PCR run in three replicates as well. The positive

control was a mock community prepared from the mixture of DNA extracted from fresh tissue samples of known vertebrates.

PCR products were resolved on a 1.2% agarose gel stained with GelRed Nucleic Acid Gel Stain (Biotum). Replicate PCR products were pooled for each sample and purified using KAPA™ Pure Beads (Kapa Biosystems Inc.), according to the manufacturer's sequencing library preparation protocol, with an elution volume of 40 µL. A second electrophoresis on a 1.2% agarose gel was run to confirm the retention of the target amplicons after purification.

A second PCR step was performed using 5 µL of purified amplicons, 1X KAPA HiFi HotStart ReadyMix (Kapa Biosystems Inc.), 10 µL of Illumina UD Indexes (Integrated DNA Technologies Inc.) and molecular biology grade water to reach a total volume of 50 µL. The following amplification conditions were used: 3 min at 95°C, 8 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, then 5 min at 72°C. A second purification was performed using KAPA™ Pure Beads (Kapa Biosystems Inc.). Purified products were resolved on a 1.2% agarose gel. Finally, the indexed libraries were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen) and pooled in equimolar concentrations in two different pools, one for each genetic marker. Both library pools were sequenced paired-end on an Illumina MiSeq System using a 300-cycle Miseq™ Micro Reagent Kit v2. The MiSeq runs were performed with a 25% PhiX v3 spike in. About 150,000 reads per sample were expected.

Bioinformatic analyses and taxonomic assignment

Reference databases creation

We created two distinct reference databases, one for each marker, by combining mitochondrial 12S rRNA sequences for all vertebrate species available in public repositories, i.e., GenBank (Benson et al., 2013) and BOLD (Ratnasingham & Hebert, 2007), using CRABS v0.1.8, an open-source software to create curated reference databases for amplicon-based sequencing (Jeunen et al., 2023).

In silico PCRs were conducted for each marker, allowing up to two mismatches in the primer-binding region. Since it is common practice to remove primer-binding regions from reference sequences when deposited in online databases, CRABS also performs a Pairwise Global Alignment (PGA) to identify and extract amplicon regions. The PGA was performed with the following settings: --speed medium --percid 0.90 --coverage 0.90 --filter_method strict. The remaining amplicon sequences were curated using a custom script to remove doubtful records and simplify records (e.g. removing records containing aff./cf./sp. in the species name and formatting hybrids records). The databases were then dereplicated to contain unique sequences per species. Environmental sequences were removed (--enviro yes), and further filtering was applied based on length (--minlen 30 --maxlen 150 for vert01; --minlen 100 --maxlen 230 for tele02) and number of missing taxonomic information (--nans 2). The two final reference databases contained 40,556 sequences for the marker

Vert01 and 26,647 sequences for the marker Tele02. The databases were finally converted into an idt-fasta format. All scripts are available at (https://github.com/giorgiastaffoni/STREAM).

Barque pipeline

Raw sequence reads were demultiplexed with bcl2fastq version 2.20 (Illumina) and quality-checked using FastQC. Retained sequences were then processed using Barque v1.8.5 (https://github.com/enormandeau/barque), an eDNA metabarcoding analysis pipeline that denoises and then annotates ASVs (Amplicon Sequence Variants) or OTUs (Operational Taxonomic Units) using high-quality reference databases. Before launching the pipeline, Tele02 and Vert01 reference databases were transformed into a Barque-friendly format (fasta header: >Family_Genus_Species) using a custom script. Subsequently, two runs were performed for each marker dataset. The first run was used to refine the database and generate denoised OTUs, along with their taxonomic assignment. In the second one, the OTUs and their taxonomic assignment were used as databases themselves to find read counts per sample (Fig. S1).

For the first run, Trimmomatic v0.39 (Bolger et al., 2014) was used for read-quality trimming with default options. Reads with lengths outside the expected range were removed, following the options set in the barque_config file (min_hit_length: 36 and crop_length: 152 for Vert01 marker; min_hit_length: 109 and crop_length: 229 for Tele02 marker). The trimmed paired-end reads were merged using Flash v1.2.11 (Magoč and Salzberg, 2011) with default options. Primers were removed allowing for 4 mismatches (20% of the primer length). Vsearch v2.22.1 (Rognes et al., 2016) was used to identify and exclude chimeric sequences with default options. To account for OTUs creation, the skip_otus option in the barque_config file was set to zero. The retained reads were compared against the custom reference databases using vsearch v2.22.1 with default options and barque_config options as follows: max_accepts: 20, max_rejects: 20 and query_cov: 0.9. We set the thresholds in the primers.csv file to assign the OTUs at species level when top matches had a percent identity above 98%; at genus level when matches were between 98% and 95%; and at family level if top matches had a percent identity lower than 95%. Sequences of species detected less than 10 times among samples were removed from the analysis (barque_config options: min_hits_sample: 10, min_hits_experiment: 10, min_size_for_otus: 10). To enhance accuracy in the taxonomic assignment and minimize erroneous sequence assignation, at the end of the first run the reference database was refined excluding the species surely not present in the study area. The pipeline was subsequently re-run with the same settings and the depleted database.

For the second run the “barque_config” and “primers” files were kept with the same parameters as in the first run, but the OTUs creation step was skipped (“barque_config” file: skip_otu= 1), and the “markers.otus.database.fasta” file from the first run was used as reference database.

Post clustering curation and decontamination

OTUs were further processed with the LULU algorithm v0.1.0, an R-package for post-clustering curation of amplicon data (Frøslev et al., 2017). The purpose of LULU is to reduce the number of erroneous OTUs to achieve more realistic biodiversity metrics by evaluating the sequence identity and co-occurrence patterns of OTUs among samples. The "marker_species_table" file resulting from our second Barque run was formatted to create an OTU_table. A match list was created using the "markers.otus.database.fasta" resulting from our first Barque run, slightly modified with an in-house generated script, and vsearch v2.21.1. As LULU was developed for ITS marker and the 12S barcode region presents lower variability, we raised the min_seq_similarity parameter to 87. The other default parameters were kept unchanged. In the resulting curated table, OTUs assigned to the same taxon were merged, and their reads were summed. OTUs with multi-hits were manually assigned to OTUs with the same taxonomic assignment selected by LULU.

Finally, data were cleaned of contaminations using microDecon v1.0.2, an R-package that uses the data in the blank samples to identify and remove the reads detected as arising from contamination (McKnight et al., 2019). Default options were applied. The replicates from the same site were then summed to obtain a single site-specific taxonomic list. Non-target species (humans and livestock) and possible contaminations were discarded. Sequences with unknown species assignments were annotated to the genus and the species was recorded as "sp.". A threshold of >10 reads was set to declare a species as present in the watercourse.

The detailed workflow (Fig. S1) and all scripts are available at the github page: https://github.com/giorgiastaffoni/STREAM. Graphical representation of the taxonomic assignment was produced using the Circlize R-package v0.4.15 (Gu et al., 2014; R Core Team, 2021) and adjusted using InkScape v1.2.2.

Results

Fish biodiversity assessment using electrofishing

We detected a total of 11 taxa, nine of which identified at species level (*Anguilla anguilla, Barbus meridionalis, Cottus gobio, Oncorhynchus mykiss, Salariopsis fluviatilis, Salmo ghigii, Salmo trutta, Squalius squalus* and *Telestes muticellus*) and two at genus level (*Salmo* sp. and *Phoxinus* sp.) (Table 1, Fig. 2). Some trout individuals were difficult to be assigned at species-level using morphological features, as they may represent hybrid individuals, which are common in the Italian trout populations. Regarding the genus *Phoxinus*, past surveys reported *P. phoxinus* (Linnaeus, 1758) as the only species present in the study area. Nowadays the distribution of the different species is under revision and not universally accepted (De Jong et al., 2014; Denys et al., 2020; De Santis et al., 2021). For this reason, a precautionary approach was adopted, considering only the genus level.

Fish communities were dominated by species belonging to the family Salmonidae in Carpasina and Tanaro, and Salmonidae and Leuciscidae in Bevera and Roia (Table 1). The Bevera and Roia rivers hosted the highest numbers of

taxa, belonging to four different families, while the Tanaro and Carpasina rivers were less rich, with only four recorded taxa (Table 1).

Sequencing outputs and taxonomic assignment

The amplicon sequencing produced 2,597,428 raw reads across 44 samples for Vert01 marker. The average (± SE) read count per sampling site was 363,095 ± 25,797, while the average read count for field negative controls was 35,334 ± 4,689. The negative PCR control resulted in 38,621 reads, while the positive control contained 97,976 reads. After the analysis with Barque, 2,141,289 sequences, belonging to 94 OTUs, were retained. Of these, 59 OTUs could be taxonomically assigned at least at order, genus or species levels. Following post-clustering curation with LULU and decontamination with microDecon, 31 OTUs (excluding the mock) were obtained. Most OTUs belonged to mammals (36%), followed by fish (32%), birds (19%), and amphibians (13%) (Table S3).

For Tele02 marker 1,673,581 raw reads across 44 samples were produced. The average (± SE) read count per sampling site was 195,094 ± 79,531. The average read count for field negative controls was 66,090 ± 51,753. Negative PCR control and positive control presented 22,808 and 17,984 reads, respectively. A total of 1,146,760 sequences belonging to 104 OTUs were retained after the analysis with Barque. Of these, 40 OTUs could be taxonomically assigned at least at order, genus or species levels. After post-clustering curation with LULU and decontamination with microDecon a total of 26 OTUs (excluding the mock) belonging to mammals (38.5%), fish (38.5%) and birds (23%) were finally identified (Table S4).

Fish biodiversity assessment using eDNA metabarcoding

Eight out of ten fish OTUs from the Tele02 assay were identified at species level (Table 2). The genera *Phoxinus* and *Salmo* had multiple matches (*P. csikii* Hankó, 1922 and *P. septimaniae* Kottelat, 2007; *S. carpio* Linnaeus, 1758, *S. cenerinus* Kottelat, 1997, *S. cettii*, *S. marmoratus* and *S. trutta*, respectively) and therefore were marked with 'sp.' (Table 2).

Seven out of ten fish OTUs obtained from the Vert01 assay were identified at species level. Some genera had multiple matches with more than one species known to occur in the area, so were marked with 'sp.', namely the genera *Barbus* (*B. barbus* Linnaeus, 1758, *B. caninus* Bonaparte, 1839 and *B. plebejus*); *Phoxinus* (*P. phoxinus, P. lumaireul* Schinz, 1840, *P. csikii* and *P. septimaniae*); *Telestes* (*T. muticellus* and *T. souffia*); and *Salmo* (*S. carpio, S. cenerinus, S. marmoratus, S. salar* Linnaeus, 1758 and *S. trutta*) (Table 2).

Six species (*Anguilla anguilla*, *Barbus plebejus, Squalius squalius, Cottus gobio, Neogobius nigricans* and *Salariopsis fluviatilis*) and two genera (*Phoxinus* and *Salmo*) were detected by both markers. Vert01 did not resolve the detection of *Barbus* sp. and *Telestes* sp. at species level, whereas Tele02 achieved a match with *Barbus meridionalis* and *Telestes*

muticellus (Fig. S2). The non detection of *Barbus meridionalis* with Vert01 was due to the lack of a barcode sequence in the reference databases of Vert01.

Overall, six orders were detected, with Cypriniformes being the dominant one and including two families: Leuciscidae and Cyprinidae. The orders Anguilliformes, Blenniiformes, Gobiiformes, and Perciformes included a species each, while Salmoniformes only included the assignation to the genus *Salmo*. Fish communities resulted dominated by Leuciscidae and Cyprinidae in the Bevera and Roia basins, which were also the most biodiverse rivers, with seven taxa identified by eDNA. On the contrary, Tanaro and Nervia were the least biodiverse with only two taxa found (Table 2).

Freshwater related vertebrate assessment using eDNA metabarcoding

Our eDNA analyses from the six sampled rivers recorded the presence of other vertebrates, among which occasional watercourses visitors. We detected birds, such as the grey heron, *Ardea cinerea*, and the dunnock, *Prunella modularis*; amphibians, such as the French cave salamander, *Speleomantes strinatii*, and the northern spectacled salamander, *Salamandrina perspicillata*; and mammals, such as the Daubenton's bat, *Myotis daubentonii*, the European edible dormouse, *Glis glis* and two water shrew species *Neomys anomalus* and *N. fodiens* (Table 3).

The marker Vert01 identified a total of 17 non-fish OTUs, of which 10 at species level, 4 at genus level and 3 at family level (Table S3). Tele02 detected a total of 14 non-fish OTUs, of which 11 at species level and 3 at family level (Table S4). Five species (*Turdus philomelos, Cervus elaphus, Glis glis, Rattus rattus, Sus scrofa*) and one family (Anatidae) were identified by both markers (Tables S3 and S4). Tele02 detected no amphibian species. Less than five non-fish species were detected in the Argentina and Nervia rivers. Carpasina and Bevera were the species richest rivers, hosting endemisms and protected taxa, such as *Salamandrina perspicillata* and *Neomys anomalus* (Tables 3, S3 and S4).

Discussion

This study validated the reliability and effectiveness of the eDNA metabarcoding approach to assess the actual fish biodiversity of rivers in Liguria, northwestern Italy. According to our findings, eDNA metabarcoding provides comparable results to electrofishing when assessing the biodiversity of Ligurian freshwater fish communities. It also allows for the detection of rare and elusive species and has a slightly higher resolution, since it was able to detect two threatened species that were not detected by traditional monitoring. Due to its many advantages, such as time and cost effectiveness and non-invasiveness, it should be considered a valid alternative to traditional monitoring methods in these environments (McColl-Gausden et al., 2021; Deiner and Altermatt, 2014; Carraro et al., 2018; Evans et al., 2017).

In the four rivers investigated using the two different monitoring techniques, six fish taxa were identified at species level by both methods (*Anguilla anguilla*, *Barbus meridionalis*, *Cottus gobio*, *Salariopsis fluviatilis*, *Squalius squalus* and *Telestes muticellus*). Both approaches were unable to identify some trout individuals (*Salmo* sp.) and the species belonging to the genus *Phoxinus*. The eDNA analysis recorded the presence of two species that were not electrofished, namely the

Italian barbel *Barbus plebejus* and the Arno goby *Neogobius nigricans*. Both species are listed in the 2022 IUCN Red List for Italy. *B. plebejus* (present in the Bevera and Roia rivers) is classified as vulnerable, and it is an Italian sub-endemism non-native to this area but reported in the Tuscano-Latium ichthyogeographic. *N. nigricans*, found in the Carpasina river, is considered endangered (Rondinini et al., 2022). This species, endemic to central Italy, was hitherto only uncertainly signalled in this area (Borroni, 2004; 2005). eDNA sampling also detected the presence of *Squalius squalus* in the Roia, where it had not been detected by electrofishing. Conversely, only a species, *Oncorhynchus mykiss*, caught by electrofishing, was not recorded with the eDNA method. This species is known to be continually introduced for fishing events, but rarely establishes stable breeding populations. (Borroni, 2004; 2005). For the two rivers investigated by eDNA monitoring only, our data represent a first effort to bridge the decades-long gap of knowledge about the species diversity of these basins. In the Nervia river, Leuciscidae represented the major family, while Cyprinidae were dominant in the Argentina (Table 2).

Most eDNA studies aimed to assess freshwater fish biodiversity use a single-marker metabarcoding approach, generally based on the fish-specific primer pairs Tele02 (Taberlet et al., 2018) or MiFish (Miya et al., 2015). Recent studies, however, showed that eDNA metabarcoding surveys designed for fish community monitoring also allow for the by-catch of non-target species (Valdez-Moreno et al., 2019; Macher et al., 2021; Mariani et al., 2021; Ritter et al., 2022). In this study, we intentionally used a multi-marker metabarcoding approach that included the analysis of Vert01 portion of mitochondrial 12S rRNA in addition to Tele02 portion to better detect the stream-associated by-catch species. Although a number of detected species were expected in the area and have little ecological relevance, some of them are listed in the 2022 IUCN Vertebrate Red List, such as the vulnerable common toad *Bufo bufo* and two water shrews *Neomys anomalus* and *N. fodiens*, both data-deficient. Of particular interest is the finding related to the occurrence of the northern spectacled salamander, *Salamandrina perspicillata*, in the Bevera river. This record falls outside the distribution range of this species, endemic to the Italian Apennines, since its western distribution limit is traditionally identified in Beigua Regional Nature Park. These results support the recent finding of a breeding population in the Ligurian Alps that had shifted the distribution limit of the species westward (Bovero et al., 2021). Further monitoring is needed to define the distribution of *S. perspicillata* in the study area.

Overall, our results confirmed that aquatic eDNA multi-marker metabarcoding is a valuable tool for monitoring Ligurian freshwater fish communities and also allow for the detection of non-target vertebrate species. Our approach was effective for the provisioning of insights into the presence of endemic, rare, threatened or allochthonous vertebrate species related to watercourses in this Italian region. It could be also critical to build ecological networks and for food web analyses, and to inform efficient management and protection schemes for Italian freshwater systems.

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Statements and Declarations

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Competing Interests

The authors declare no competing interests.

Author contributions

Lorenzo Ballini, Giorgia Staffoni, Alessio Iannucci and Sara Fratini wrote the manuscript. Sara Fratini and Alessio Iannucci conceptualized and designed the study. Lorenzo Ballini collected the eDNA samples. Dario Ottonello, Alessandro Candiotto, Simone Forte and Paolo Vezza conducted the electrofishing survey. Lorenzo Ballini, Alessio Iannucci and Sara Fratini performed lab work and provided lab support. Giorgia Staffoni ran the bioinformatics analysis, with the support of Davide Nespoli. All authors read and approved the final version of the manuscript.

Data availability

The detailed workflow and all scripts used in this study are available at the github page: https://github.com/giorgiastaffoni/STREAM. Raw reads have been deposited in the NCBI Short Read Archive (accession no. pending).

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Tables

Table 1 Fish community compositions identified by the 2022 electrofishing sampling campaigns.

Order	**Family**	**Species**	Roia	Bevera	Carpasina	Tanaro
Anguilliformes	Anguillidae	*Anguilla anguilla* (Linnaeus, 1758)	×	×		
Blenniiformes	Blenniidae	*Salariopsis fluviatilis* (Asso, 1801)	×			
Cypriniformes	Cyprinidae	*Barbus meridionalis* Risso, 1827	×	×		
Cypriniformes	Leuciscidae	*Phoxinus* sp.	×	×		
Cypriniformes	Leuciscidae	*Squalius squalus* (Bonaparte, 1837)		×		
Cypriniformes	Leuciscidae	*Telestes muticellus* (Bonaparte, 1837)	×	×	×	
Perciformes	Cottidae	*Cottus gobio* Linnaeus, 1758				×
Salmoniformes	Salmonidae	*Oncorhynchus mykiss* (Walbaum, 1792)	×		×	×
Salmoniformes	Salmonidae	*Salmo ghigii* Pomini, 1941		×		
Salmoniformes	Salmonidae	*Salmo* sp.		×	×	×
Salmoniformes	Salmonidae	*Salmo trutta* Linnaeus, 1758	×	×	×	×

Table 2 Fish communities composition inferred by the eDNA metabarcoding monitoring. Data of presence/absence were obtained by combining the results of the two 12S rRNA mitochondrial barcoding markers Tele02 and Vert01. Asterisks indicate the four sites for which electrofishing data are available.

Order	**Family**	**Species**	Roia*	Bevera*	Carpasina*	Tanaro*	Argentina	Nervia
Anguilliformes	Anguillidae	*Anguilla anguilla* (Linnaeus, 1758)	×	×			×	×
Blenniiformes	Blenniidae	*Salariopsis fluviatilis* (Asso, 1801)	×					
Cypriniformes	Cyprinidae	*Barbus meridionalis* Risso, 1827	×	×				
Cypriniformes	Cyprinidae	*Barbus plebejus* Bonaparte, 1839	×	×			×	
Cypriniformes	Leuciscidae	*Phoxinus* sp.	×	×				
Cypriniformes	Leuciscidae	*Squalius squalus* (Bonaparte, 1837)	×	×				
Cypriniformes	Leuciscidae	*Telestes muticellus* (Bonaparte, 1837)	×	×	×		×	×
Gobiiformes	Gobiidae	*Neogobius nigricans* (Canestrini, 1867)			×			
Perciformes	Cottidae	*Cottus gobio* Linnaeus, 1758				×		
Salmoniformes	Salmonidae	*Salmo* sp.		×	×	×		

Table 3 Non-fish vertebrate taxa identified at species or genus level per site from freshwater eDNA metabarcoding. Data of presence/absence were obtained by combining the results of the two 12S rRNA mitochondrial barcoding markers Tele02 and Vert01.

Group	**Family**	**Species**	Argentina	Roia	Tanaro	Carpasina	Bevera	Nervia
Amphibians	Bufonidae	*Bufo bufo* (Linnaeus, 1758)		x	x	x	x	x
	Plethodontidae	*Speleomantes strinatii* (Aellen, 1958)				x		
	Ranidae	*Pelophylax* sp.		x	x		x	
	Salamandridae	*Salamandrina perspicillata* (Savi, 1821)					x	
Birds	Ardeidae	*Ardea cinerea* Linnaeus, 1758		x				
	Fringillidae	*Fringilla* sp.	x		x			
	Phalacrocoracidae	*Phalacrocorax carbo* (Linnaeus, 1758)		x				
	Prunellidae	*Prunella modularis* Linnaeus, 1758				x		
	Turdidae	*Turdus philomelos* C.L. Brehm, 1831	x			x	x	x
Mammals	Canidae	*Canis lupus* Linnaeus, 1758				x	x	
	Cervidae	*Cervus elaphus* Linnaeus, 1758					x	
	Felidae	*Felis* sp.				x		
	Gliridae	*Glis glis* (Linnaeus, 1766)				x		
	Muridae	*Apodemus sylvaticus* (Linnaeus, 1758)		x				
	Muridae	*Rattus rattus* (Linnaeus, 1758)				x		x
	Soricidae	*Neomys anomalus* Cabrera, 1907				x		
	Soricidae	*Neomys fodiens* (Pennant, 1771)			x			
	Suidae	*Sus scrofa* Linnaeus, 1758		x	x	x	x	x
	Vespertilionidae	*Myotis daubentonii* (Kuhl, 1817)		x				

Figure captions

Fig. 1 Map of the study area. Bullets indicate eDNA sampling sites (orange) and sampling sites where also electrofishing monitoring was conducted (red).

Fig. 2 *Circlize* plot showing fish species identified by environmental DNA metabarcoding, electrofishing, or both methods. For the genus *Salmo*, all the species were combined into a single record *Salmo* sp..

Supplementary material

Supplementary material: Table S1, S2, S3, S4 and Figure S1, S2.

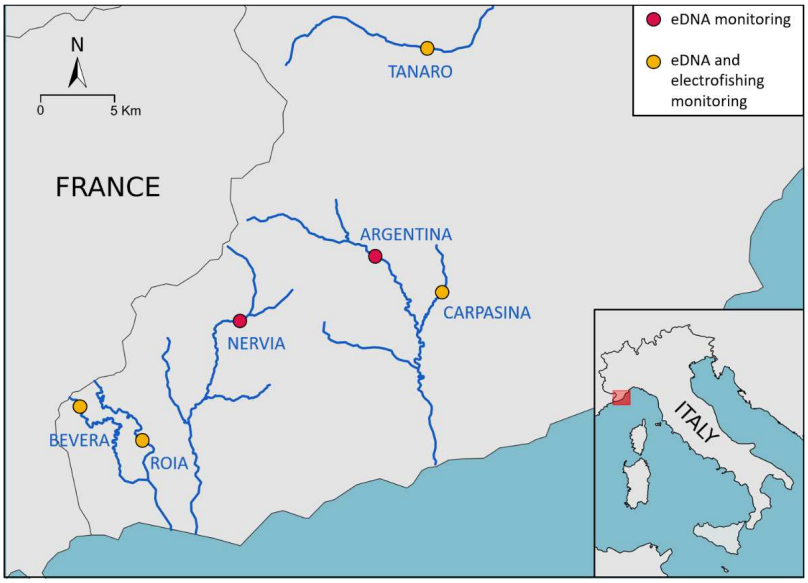
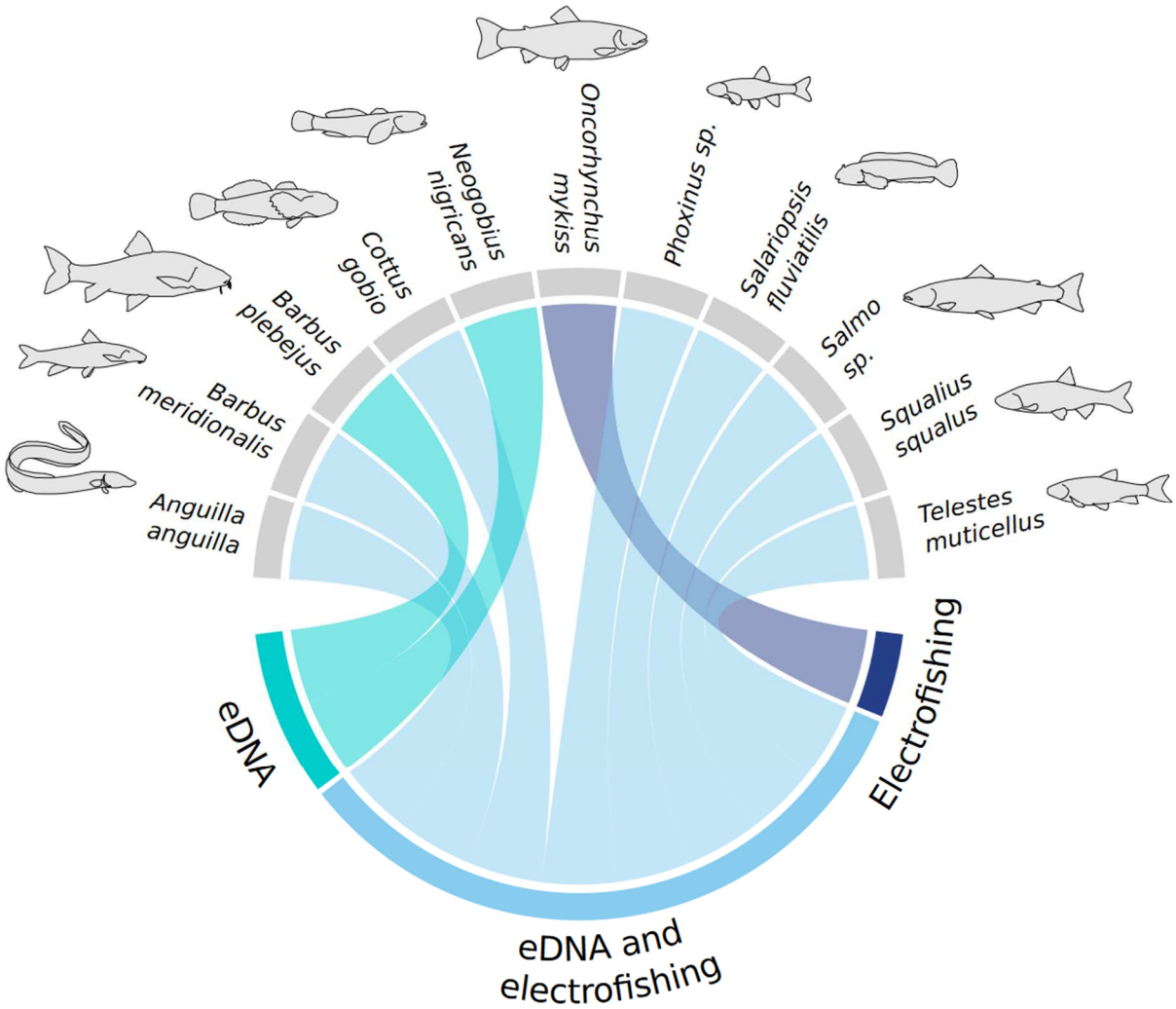

eDNA monitoring
eDNA and electrofishing monitoring
N
0
5 Km
FRANCE
TANARO
ARGENTINA
CARPASINA
NERVIA
BEVERA
ROIA
ITALY

Fig. 1.



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558 **Fig. 2**

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