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1 **Environmental DNA metabarcoding as an efficient tool to monitor freshwater systems in**
2 **northwestern Italy**

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15

16 **Abstract**

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

29 **Keywords**

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

31 **Introduction**

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

42 A prerequisite for the development of protection policies of habitats and native species is to perform reliable monitoring
43 surveys, which allow the assessment of the species richness within an ecosystem and the conservation status of the
44 different species (Aglieri et al., 2021; Seymour et al., 2021). Traditional methods for biodiversity monitoring are based
45 on visual observation or capture-based samplings, which are both time consuming and have recognised limitations such
46 as identification errors, human disturbance on the habitat and other difficulties of application (Lacoursière-Roussel et al.,
47 2016; Deiner et al., 2017). In recent years, environmental DNA (eDNA) analysis has been confirmed as an innovative
48 biodiversity monitoring tool and has been widely used, with a special focus on aquatic environments (Hänfling et al.,
49 2016; Shaw et al., 2016; Seymour et al., 2021; Schenekar, 2023). The eDNA approach is more sensitive than the above-
50 mentioned traditional methods, since it allows early detection of rare and invasive species, as well as juvenile and cryptic
51 stages of organisms (Evans et al., 2017; Thomsen & Willerslev, 2015; Ballini et al., 2024). It also proved to be a non-
52 invasive and cost-effective method (Evans et al., 2017; Taberlet et al., 2018; Pawlowski et al., 2020). This approach has
53 been applied in monitoring programmes serving various purposes, such as species identification, species richness
54 estimation, community monitoring and development of biotic indexes (Ruppert et al., 2019).

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

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

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

76 **Material and Methods**

77 **Study area**

78 The sampling was performed at six rivers in western Liguria (northwestern Italy): Roia, Bevera, Argentina, Carpasina,
79 Nervia and Tanaro (Fig. 1). The Tanaro basin is located at the border of the Padano-Venetian ichthyogeographic district,
80 while the other watercourses flow into the Ligurian Sea. Parts of the Roia and Bevera rivers are located in France (Bianco,
81 1990; 1995). Based on long-term traditional monitoring programs and historical data, approximately 12 fish species occur
82 in these drainage basins, most of which belong to the families Leuciscidae (*sensu* Schönhuth et al., 2018), Cyprinidae and
83 Salmonidae (Table S1) (Borroni, 2004; Borroni, 2005; Ciuffardi, 2006; Ciuffardi et al., 2015). The area is home to several
84 endemic and threatened vertebrate species according to the IUCN, such as the critically endangered European eel, the
85 data deficient freshwater blenny, the near threatened dunnock and the vulnerable common toad.

86 **Electrofishing**

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

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

93 **eDNA sampling**

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

105 **DNA extraction, library amplification and sequencing**

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

110 Environmental DNA was extracted from membrane filters using the ZymoBIOMICS™ DNA Miniprep Kit according to
111 the manufacturer's instructions. DNAs were eluted in a final volume of 50 µL of sterilised water and then stored at -25
112 °C. After the purification, the DNA extracted from each sample was quantified using the Invitrogen™ Qubit™ 4
113 Fluorometer and the Invitrogen™ Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific Inc.).

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

116 Amplicon libraries were prepared following the dual-PCR protocol for Illumina platforms (Bourlat et al., 2016). In the
117 first PCR step, barcode markers were amplified in a total volume of 25 µL using 1X Invitrogen™ Taq DNA Polymerase
118 PCR Buffer, 1 U of Taq DNA Polymerase (Thermo Fisher Scientific Inc.), 2 mM MgCl₂, 0.3 mM dNTPs, 0.5 µM of each
119 primer enriched with an Illumina adapter overhang (Table S2) and 1 µL of template DNA. The following amplification
120 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
121 1 min, then 10 min at 72°C. For each sampling site, the amplification of eDNA samples was performed on three technical
122 replicates. Negative field controls, an amplification negative control (containing DNA-free ddH₂O instead of template
123 DNA) and an amplification positive control were included in the same PCR run in three replicates as well. The positive

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

126 PCR products were resolved on a 1.2% agarose gel stained with GelRed Nucleic Acid Gel Stain (Biotum). Replicate PCR
127 products were pooled for each sample and purified using KAPA™ Pure Beads (Kapa Biosystems Inc.), according to the
128 manufacturer's sequencing library preparation protocol, with an elution volume of 40 µL. A second electrophoresis on a
129 1.2% agarose gel was run to confirm the retention of the target amplicons after purification.

130 A second PCR step was performed using 5 µL of purified amplicons, 1X KAPA HiFi HotStart ReadyMix (Kapa
131 Biosystems Inc.), 10 µL of Illumina UD Indexes (Integrated DNA Technologies Inc.) and molecular biology grade water
132 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
133 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
134 Beads (Kapa Biosystems Inc.). Purified products were resolved on a 1.2% agarose gel. Finally, the indexed libraries were
135 quantified using the Qubit dsDNA HS Assay Kit (Invitrogen) and pooled in equimolar concentrations in two different
136 pools, one for each genetic marker. Both library pools were sequenced paired-end on an Illumina MiSeq System using a
137 300-cycle Miseq™ Micro Reagent Kit v2. The MiSeq runs were performed with a 25% PhiX v3 spike in. About 150,000
138 reads per sample were expected.

139 **Bioinformatic analyses and taxonomic assignment**

140 *Reference databases creation*

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

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

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

156 *Barque pipeline*

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

165 For the first run, Trimmomatic v0.39 (Bolger et al., 2014) was used for read-quality trimming with default options. Reads
166 with lengths outside the expected range were removed, following the options set in the barque_config file
167 (min_hit_length: 36 and crop_length: 152 for Vert01 marker; min_hit_length: 109 and crop_length: 229 for Tele02
168 marker). The trimmed paired-end reads were merged using Flash v1.2.11 (Magoč and Salzberg, 2011) with default
169 options. Primers were removed allowing for 4 mismatches (20% of the primer length). Vsearch v2.22.1 (Rognes et al.,
170 2016) was used to identify and exclude chimeric sequences with default options. To account for OTUs creation, the
171 skip_otus option in the barque_config file was set to zero. The retained reads were compared against the custom reference
172 databases using vsearch v2.22.1 with default options and barque_config options as follows: max_accepts: 20,
173 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
174 top matches had a percent identity above 98%; at genus level when matches were between 98% and 95%; and at family
175 level if top matches had a percent identity lower than 95%. Sequences of species detected less than 10 times among
176 samples were removed from the analysis (barque_config options: min_hits_sample: 10, min_hits_experiment: 10,
177 min_size_for_otus: 10). To enhance accuracy in the taxonomic assignment and minimize erroneous sequence assignment,
178 at the end of the first run the reference database was refined excluding the species surely not present in the study area.
179 The pipeline was subsequently re-run with the same settings and the depleted database.

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

183 *Post clustering curation and decontamination*

184 OTUs were further processed with the LULU algorithm v0.1.0, an R-package for post-clustering curation of amplicon
185 data (Frøslev et al., 2017). The purpose of LULU is to reduce the number of erroneous OTUs to achieve more realistic
186 biodiversity metrics by evaluating the sequence identity and co-occurrence patterns of OTUs among samples. The
187 “marker_species_table” file resulting from our second Barque run was formatted to create an OTU_table. A match list
188 was created using the “markers.otus.database.fasta” resulting from our first Barque run, slightly modified with an in-
189 house generated script, and vsearch v2.21.1. As LULU was developed for ITS marker and the 12S barcode region presents
190 lower variability, we raised the min_seq_similarity parameter to 87. The other default parameters were kept unchanged.
191 In the resulting curated table, OTUs assigned to the same taxon were merged, and their reads were summed. OTUs with
192 multi-hits were manually assigned to OTUs with the same taxonomic assignment selected by LULU.
193 Finally, data were cleaned of contaminations using microDecon v1.0.2, an R-package that uses the data in the blank
194 samples to identify and remove the reads detected as arising from contamination (McKnight et al., 2019). Default options
195 were applied. The replicates from the same site were then summed to obtain a single site-specific taxonomic list. Non-
196 target species (humans and livestock) and possible contaminations were discarded. Sequences with unknown species
197 assignments were annotated to the genus and the species was recorded as “sp.”. A threshold of >10 reads was set to
198 declare a species as present in the watercourse.
199 The detailed workflow (Fig. S1) and all scripts are available at the github page:
200 <https://github.com/giorgiastaffoni/STREAM>. Graphical representation of the taxonomic assignment was produced using
201 the Circlize R-package v0.4.15 (Gu et al., 2014; R Core Team, 2021) and adjusted using InkScape v1.2.2.

202 **Results**

203 **Fish biodiversity assessment using electrofishing**

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

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

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

216 **Sequencing outputs and taxonomic assignment**

217 The amplicon sequencing produced 2,597,428 raw reads across 44 samples for Vert01 marker. The average (\pm SE) read
218 count per sampling site was $363,095 \pm 25,797$, while the average read count for field negative controls was $35,334 \pm$
219 $4,689$. The negative PCR control resulted in 38,621 reads, while the positive control contained 97,976 reads. After the
220 analysis with Barque, 2,141,289 sequences, belonging to 94 OTUs, were retained. Of these, 59 OTUs could be
221 taxonomically assigned at least at order, genus or species levels. Following post-clustering curation with LULU and
222 decontamination with microDecon, 31 OTUs (excluding the mock) were obtained. Most OTUs belonged to mammals
223 (36%), followed by fish (32%), birds (19%), and amphibians (13%) (Table S3).

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

231 **Fish biodiversity assessment using eDNA metabarcoding**

232 Eight out of ten fish OTUs from the Tele02 assay were identified at species level (Table 2). The genera *Phoxinus* and
233 *Salmo* had multiple matches (*P. csikii* Hankó, 1922 and *P. septimaniae* Kottelat, 2007; *S. carpio* Linnaeus, 1758, *S.*
234 *cenerinus* Kottelat, 1997, *S. cettii*, *S. marmoratus* and *S. trutta*, respectively) and therefore were marked with 'sp.' (Table
235 2).

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

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

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

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

251 **Freshwater related vertebrate assessment using eDNA metabarcoding**

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

257 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
258 level (Table S3). Tele02 detected a total of 14 non-fish OTUs, of which 11 at species level and 3 at family level (Table
259 S4). Five species (*Turdus philomelos*, *Cervus elaphus*, *Glis glis*, *Rattus rattus*, *Sus scrofa*) and one family (Anatidae) were
260 identified by both markers (Tables S3 and S4). Tele02 detected no amphibian species. Less than five non-fish species
261 were detected in the Argentina and Nervia rivers. Carpasina and Bevera were the species richest rivers, hosting endemisms
262 and protected taxa, such as *Salamandrina perspicillata* and *Neomys anomalus* (Tables 3, S3 and S4).

263 **Discussion**

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

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

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

286 Most eDNA studies aimed to assess freshwater fish biodiversity use a single-marker metabarcoding approach, generally
287 based on the fish-specific primer pairs Tele02 (Taberlet et al., 2018) or MiFish (Miya et al., 2015). Recent studies,
288 however, showed that eDNA metabarcoding surveys designed for fish community monitoring also allow for the by-catch
289 of non-target species (Valdez-Moreno et al., 2019; Macher et al., 2021; Mariani et al., 2021; Ritter et al., 2022). In this
290 study, we intentionally used a multi-marker metabarcoding approach that included the analysis of Vert01 portion of
291 mitochondrial 12S rRNA in addition to Tele02 portion to better detect the stream-associated by-catch species. Although
292 a number of detected species were expected in the area and have little ecological relevance, some of them are listed in the
293 2022 IUCN Vertebrate Red List, such as the vulnerable common toad *Bufo bufo* and two water shrews *Neomys anomalus*
294 and *N. fodiens*, both data-deficient. Of particular interest is the finding related to the occurrence of the northern spectacled
295 salamander, *Salamandrina perspicillata*, in the Bevera river. This record falls outside the distribution range of this species,
296 endemic to the Italian Apennines, since its western distribution limit is traditionally identified in Beigua Regional Nature
297 Park. These results support the recent finding of a breeding population in the Ligurian Alps that had shifted the distribution
298 limit of the species westward (Bovero et al., 2021). Further monitoring is needed to define the distribution of *S.*
299 *perspicillata* in the study area.

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

305

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309 **Statements and Declarations**

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316

317 **Competing Interests**

318 The authors declare no competing interests.

319

320 **Author contributions**

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

326 **Data availability**

327 The detailed workflow and all scripts used in this study are available at the github page:
328 <https://github.com/giorgiastaffoni/STREAM>. Raw reads have been deposited in the NCBI Short Read Archive (accession
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330

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525 **Tables**526 **Table 1** Fish community compositions identified by the 2022 electrofishing sampling campaigns.

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

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531 **Table 2** Fish communities composition inferred by the eDNA metabarcoding monitoring. Data of presence/absence were
 532 obtained by combining the results of the two 12S rRNA mitochondrial barcoding markers Tele02 and Vert01. Asterisks
 533 indicate the four sites for which electrofishing data are available.
 534

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

535

536 **Table 3** Non-fish vertebrate taxa identified at species or genus level per site from freshwater eDNA metabarcoding. Data
 537 of presence/absence were obtained by combining the results of the two 12S rRNA mitochondrial barcoding markers
 538 Tele02 and Vert01.
 539

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

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542 **Figure captions**

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

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

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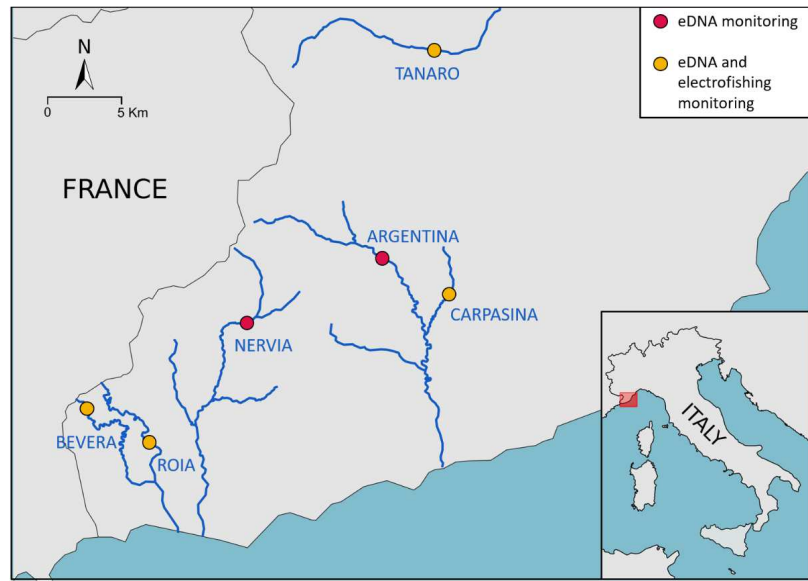
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549 **Supplementary material**

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

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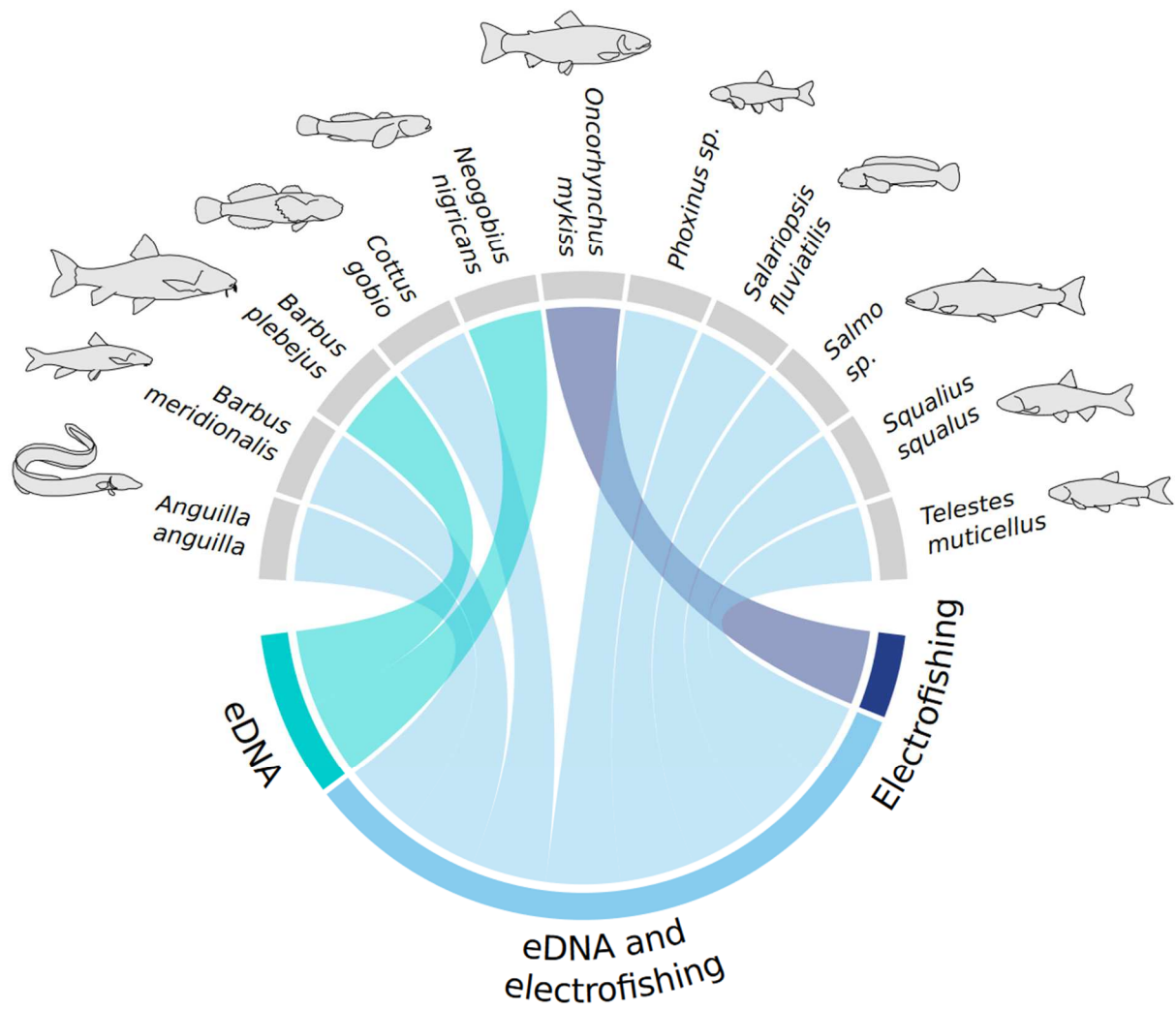
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Fig. 1.



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Fig. 2