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Efficient eDNA-based assessment of mitochondrial lineage diversity in wild brown trout populations

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Among the numerous applications of environmental DNA (eDNA), the assessment of genetic diversity in wild populations of target taxa remains moderately investigated. Focusing on 10 selected sites inhabited by brown trout (*Salmo trutta* complex) in central Italian watercourses, we: (1) developed a non-invasive genetic monitoring protocol combining environmental DNA sampling, amplicon sequencing with custom-designed primers targeting a 192 bp fragment of the mitochondrial Control Region (CR) – whose evolutionary lineages are informative for brown trout phylogeography and genetic conservation status—and a curated reference database; (2) validate it comparing CR lineages assemblages revealed by eDNA with those obtained through traditional CR genotyping of trouts collected by electrofishing in recent surveys, while accounting for uncertainty associated with sample size of genotyped individuals. The two methods returned strongly correlated per-site raw frequencies of CR lineages (Pearson $r = 0.93$, $p < 0.0001$), and remarkably similar lineage assemblages (Mantel $r = 0.832$, $p = 0.0001$). Concordances between (semi-quantitative) lineage assemblages retrieved by the two methods increased at increasing trout densities and decreased at warmer water temperatures. Discordances mostly involved low-frequency lineages that were not detected by traditional CR genotyping. This study supports the effectiveness of eDNA for reliably characterising mitochondrial intra-population diversity in brown trout, a widely distributed and actively managed taxon of important conservation and economic value, offering a valuable tool to assist the genetic monitoring of wild populations, including the identification and quantification of exotic lineages.

Keywords *Salmo trutta* complex, Evolutionary control region lineages, Environmental DNA, Genetic monitoring, Next-generation amplicon sequencing, Freshwaters

Environmental DNA (eDNA) refers to genomic DNA, either fragmented or relatively intact, that organisms shed into environmental matrices (water, air, soil or sediments) through excretions, mucous, sloughed tissues, faeces[1,2]. Among its broad range of applications[3], eDNA sampling coupled with next-generation amplicon sequencing has been widely and successfully used to explore community ecology and to detect or monitor fish species[4], in both marine and freshwater ecosystems (e.g[5–7]). Studies provided compelling evidence for a positive association between proxies of eDNA amount and (relative) species abundance or biomass, which is reportedly influenced by environmental factors in some cases (reviewed in[8]).

Although still relatively unexplored, the analysis of eDNA – either targeting single or multiple taxa – may also provide information on intraspecific diversity and population genetics[9–11]. Importantly, genetic variation captured through eDNA reflects an aggregate signal at the population level, comprising a pool of haplotypes/alleles from an unknown number of genetic contributors[11]. Despite such limitations, eDNA-based haplotyping has been employed to trace the dispersal of the freshwater exotic invasive *Lepomis macrochirus* in Japan[12], or to infer the number of *Anguilla anguilla* individuals through haplotype count in both an experimental tank and three Norwegian watercourses[13]. In an innovative way[14], successfully obtained individual mitochondrial haplotypes by collecting eDNA nearby from wild whale sharks of the Australian Ningaloo Reef, as verified through traditional tissue genotyping.

Basically, the analysis of eDNA diversity can potentially assist genetic assessments and enhance non-invasive monitoring of wild populations, while reducing costs and time compared to traditional methods[9,15].

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This is particularly relevant for taxa of noteworthy economic and conservation value, as well as for alien invasive species[16]. All these features are typical of brown trout, an iconic and extensively studied Salmonid native to the Western Palearctic area, showing extraordinary ecological and morphological plasticity, along with remarkable genetic polymorphism[17], and is therefore commonly referred to as *Salmo trutta* species complex[18]. Although brown trout taxonomy and systematics remain controversial, early phylogenetic studies analysing mitochondrial Control Region (CR) sequences recognized multiple evolutionary lineages/haplogroups (hereafter simply "lineages"), often inconsistent with ecomorphological forms, whose differentiation started during the Pliocene[19] or possibly earlier[20]. To date, five relatively widespread major lineages – Adriatic (AD), Atlantic (AT), Danubian (DA), *marmoratus* (MA), Mediterranean (ME) – plus a few geographically restricted minor ones have been described. Lineages may co-occur in native populations as their spatial distribution partly overlaps in the Mediterranean area, suggesting an intricate evolutionary history and phylogeographic pattern in brown trout[17,18].

Brown trout has been intensively managed since the last century because of its economic value as angling and food resources: anthropogenic introductions in ecologically-suitable areas and massive stocking have been reiterated at the global scale, causing a range expansion and various (genetic) alterations within populations[21,22]. Importantly, stocking activities have been mostly achieved using hatchery strains of Atlantic origin belonging to the AT lineage, often without considering the genetic complexity of the receiving wild populations[23] (and references therein). Since the AT lineage naturally occurred in European watercourses flowing into the Atlantic Ocean, its presence in wild populations within the Mediterranean area proves the release of (exotic) hatchery trout. In fact, despite the genomic revolution, brown trout lineages (and their haplotypes) are still routinely employed for preliminary, rapid, unambiguous and easily replicable characterization of genetic makeup and conservation status in wild populations[24–27], being particularly convenient for large-scale conservation-oriented projects (e.g[28]).

Several studies investigated the effectiveness of barcoding/metabarcoding approaches based on eDNA-collection for brown trout detection and monitoring in the wild[29–31], particularly in introduction areas where it represents a threat to native species – note that Atlantic brown trout is listed among "100 of the World's worst invasive alien species"[32]. Other studies employed brown trout as a model to disentangle patterns of eDNA dispersal[33], compare the efficiency of filtration methods for eDNA quantification[34], or assess the relationship between eDNA concentration and trout abundance while controlling for the effect of environmental/laboratory factors in natural conditions[35]. However, to the best of our knowledge, eDNA has not yet been used to assess mitochondrial diversity and conservation status in brown trout populations.

In this study, we developed and validated an eDNA-based protocol to assess brown trout mitochondrial (lineage) diversity in wild populations. Our approach relies on next-generation amplicon sequencing of a lineage-diagnostic fragment of the CR, amplified with specifically designed primers (Fig. 1). We tested this method in 10 environmentally diverse sampling sites from central Italy, a hotspot of brown trout genetic diversity, where up to four evolutionary lineages (AD, ME, MA and AT) co-occur in wild resident populations of either native, admixed or alien-stocked origin, as revealed by recent traditional genetic assessments[25,36]. Finally, we evaluated whether the concordance between lineage assemblages inferred from eDNA and traditional assessments correlated with demographic or environmental variables.

Materials and methods

Sampling design

For eDNA sampling, which was carried out in 2024 (see below), we selected 10 sites in central Italian watercourses characterized by: (1) the presence of brown trout populations recently characterized at the mitochondrial CR with an adequate number of individuals – these eventually provided reference frequencies for the present study (see the "Data analyses" section); (2) hosting multiple brown trout lineages, either native or exotic; (3) heterogeneous habitat conditions in terms of altitude, water temperature and water flow (Table 1). In summary, the chosen sites included cold-water creeks and wadable streams, from lowland to mountain areas, in which 1–4 CR lineages were found by traditional genotyping – namely DNA extraction from tissue samples, PCR-amplification and Sanger sequencing of CR from 14 to 30 trout/per site, randomly captured by electrofishing during summer 2018 or 2019 (Table 1; Fig. 1).

eDNA collection and estimates of brown trout abundance

The workflow is visually summarized in Fig. 1. We first collected eDNA by filtering a total of 4 L of stream water (two replicates of 2 L each collected ~ 20 m apart) directly in the watercourses, using a vacuum pump and the sterile filters containing a 0.45 µm mesh membrane of the eDNA Citizen Scientist Kit (Smith-Root; https://www.smith-root.com/edna/edna-citizen-scientist-sampler). To account for possible contaminations from filtering operations, we included a field blank by filtering two litres of sealed bottled drinking water. Successively, we followed the two-pass removal electrofishing[37,38] over stretches of approximately 10–20 times the riverbed width, and annotated the number of captured trout and their cumulative biomass for each pass separately. Finally, we recorded water temperature and water flow. To minimize (cross)contamination, operators wore disposable face masks and nitrile gloves during sampling, and fishing equipment was sterilised with ~ 15% bleach solution between sampling sessions. Sampling protocols followed standard procedures and were approved by the "Direzione agricoltura, promozione della filiera e della cultura del cibo, caccia e pesca, foreste" of the Latium Region (authorization no. G07347 of 8/6/2022) and by the Parco Naturale Regionale dei Monti Simbruini (no. 2052 of 25/06/2024).

For the 2024 sampling, we inferred trout abundance as both standing crop (SC_{2024}) and density index (DI_{2024}), which are, respectively, the biomass and number of individuals estimated by the two-pass removal method[37,38], divided by the sampling surface to obtain comparable measures (Table 1). Note that the 2024 sampling (during

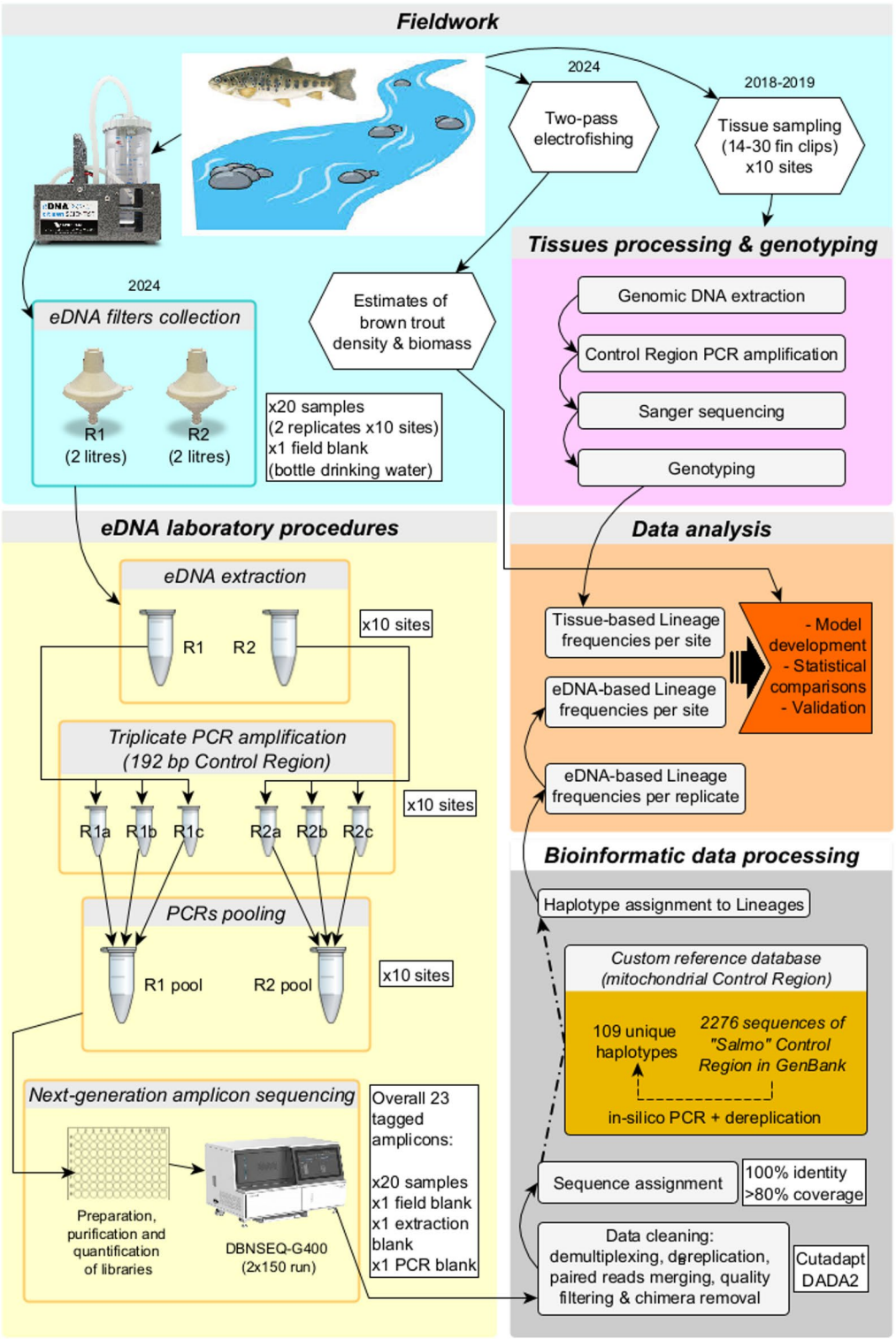

Fig. 1. Workflow illustrating rationale and major steps of eDNA collection, laboratory procedures, data processing and result validation in this study.

Sampling site	Catchment	Latitude (North)	Longitude (East)	Altitude [m a.s.l.]	Water flow [L/s]	Water temperature [°C]	SC_{2024} [g/m^2]	DI_{2024} [individuals/m^2]	Year of tissue sampling	# tissue samples	# CR lineages	Data source
CAR	Carpello	41.7	13.7	309	849	10.7	3.59	0.11	2019	30	1	[36]
COS	Cosa	41.8	13.4	693	375	10.9	6.86	0.35	2019	20	2	Unpublished data
MOL	Molinaro	42.6	13.3	837	38	15.3	9.06	0.08	2018	26	2	[25]
PES	Pescara	42.7	13.2	780	122	12.8	10.68	0.58	2019	25	2	Unpublished data
RAP	Rapido	41.5	13.9	120	276	14.3	4.45	0.08	2019	30	2	[36]
RAT	Ratto	42.5	13.1	848	45	12.6	38.65	1.02	2018	25	2	[25]
SCR	Santacroce	41.3	13.7	48	283	12.8	30.25	0.28	2019	30	2	[36]
SIM1	Simbrivio	41.9	13.2	800	205	10.8	14.35	0.15	2019	16	2	[36]
SIM2	Simbrivio	41.9	13.2	694	420	12.9	19.46	0.41	2019	14	3	[36]
TRO	Tronto	42.7	13.3	665	1344	15.9	10.33	0.10	2019	25	4	[36]

Table 1. Environmental and demographic information of 10 brown trout sites gathered in 2024 sampling – site code and catchment, geographical coordinates (datum = WGS84), altitude, physical water parameters and trout abundance measures (SC_{2024} = standing crop, DI_{2024} = density index) – plus overview on previous genetic assessments – sampling year, tissue sample size and the number of detected lineages with the corresponding reference source.

which we collected eDNA samples and obtained the above-mentioned trout abundance estimates) and 2018–2019 sampling (during which we collected tissue samples) were carried out exactly in the same river stretches, to minimize possible discrepancies due to genetic (sub)structuring in wild brown trout populations[36,39–41].

Custom primer design and cross-amplification tests

With Primer3Plus[42], we designed a new forward primer (5'-GCAAAACACGTGATAATAACCAA-3') that, combined with the reverse primer HDL-C-1 (5'-CCTGAAGTAGGAACCAGATGCCAG-3'; successfully employed for *S. trutta* by[43]), targeted a 191–193 base pairs (bp) fragment of the brown trout mitochondrial CR, as verified through an in silico PCR (≤ 4 bp primer mismatches) with the Basic Local Alignment Search Tool (BLAST)[44]. This CR fragment (1) was sufficiently short to ensure amplification of likely fragmented eDNA[1], and (2) included diagnostic nucleotides for major *Salmo trutta* complex evolutionary lineages in the Mediterranean and Atlantic area (AD, AT, MA, ME, DA), plus the Duero (DU) and North-African (NA) geographically restricted lineages[17]. An in-silico PCR with relaxed settings (≤ 6 bp primer mismatches) was also performed to evaluate cross-amplification in Salmonids, revealing probable amplification in multiple *Salmo* taxa (including phylogenetically basal *S. salar*, *S. obtusirostris* and *S. ohridanus*[17]) and other genera (*Oncorhynchus*, *Parahucho*, *Salvelinus*, *Salvethymus*). However, sequence differences in the amplified CR fragment allow for easy distinguishing of taxa (haplotype neighbour-net network in Figure S1). Sanger sequencing of PCR products from 5x *S. trutta*, 2x *O. mykiss*, and 2x *S. salar* tissue samples confirmed amplification of the target CR fragment (100% match with multiple GenBank-deposited sequences), and excluded unintentional amplification of nuclear/mitochondrial pseudogenes.

DNA extraction, library preparation and sequencing

Collected filters were individually stored at −20 °C in plastic bags until eDNA extraction. We extracted eDNA from half of the membrane encapsulated within each filter following the tissue protocol of the Quick DNA Miniprep kit (Zymo Research), and eluted the total DNA in 50 µL of the elution buffer. We also included an extraction blank (containing only reagents) to control for possible contamination associated with extraction procedures and reagents.

We PCR-amplified the brown trout CR fragment using the primers described above, each including an 8 bp tag at the 5' end. Tags were unique to each sample and identical for both forward and reverse primers, with tag sequences differing by at least 3 bp to minimize tag jumping and sequence-to-sample misidentifications[1,45].

Overall, 20 samples and three controls – a field blank, plus an extraction and a PCR blank, as all samples were processed altogether – were each amplified in triplicate using a single-step PCR protocol[1]. PCR reactions were carried out in a TC9610 Multigene OptiMax Thermal Cycler in a total volume of 20 µL, consisting of: 10 µL of 2× AmpliTaqGold™360MasterMix (Life Technologies); 1 µL of each primer (10 µM); 2 µL of template DNA (~ 10–100 ng); 6 µL of PCR-grade water. PCR conditions consisted of an initial denaturation/polymerase activation step at 95 °C for 10', followed by 40 cycles at 95 °C for 30", 54 °C annealing for 1', 72 °C for 30", and a final elongation step at 72 °C for 7'. We verified amplification success by visualizing PCR products on a 2% agarose gel electrophoresis using a UV transilluminator. Subsequently, we pooled the three PCR replicates per sample (Fig. 1) – the same procedure was applied to the control samples, which showed no amplification signal on the transilluminator.

After quantification with Qubit™ Flex Fluorometer (ThermoFisher), amplicons were pooled equimolarly. The pooled sample was quality checked (i.e., peak distribution and concentration) using a 4150 TapeStation System (Agilent), and subsequently purified using MagSi-NGS^{PREP} Plus magnetic beads (magtivio) to remove non-target DNA fragments. A second round of quantification and quality check was performed before sending the

purified pool to an external service provider (BGI Genomics, https://www.bgi.com/global) for next-generation paired-end sequencing (PE150) on a DNBSEQ-G400 platform.

Reads processing, custom reference database construction and variant assignment

Demultiplexing of raw amplicon sequencing reads and tag removal were performed using Cutadapt v5.0[46]. Reads were processed in QIIME2 v2024.10.1[47] using the implemented DADA2 pipeline[48] with default settings. This allowed primer sequence removal, paired reads merging, variant dereplication and denoising, and chimaera removal. Obtained variants were compared against a custom CR reference database (see below) for the assignments to known haplotypes and thus lineages. Assignments (BLAST + classification algorithm[49]) were made at a 100% identity threshold to prevent misidentification of lineages differing by a single mutation within the target sequence (Fig. 1).

The custom reference database was built with CRABS v1.0.0[50]. In brief, we: (1) comprehensively downloaded all CR sequences of the *Salmo* genus available in NCBI nucleotide database on 1 March 2025; (2) performed an in silico PCR with our primer pair (default parameters) to retain only target sequence fragments; (3) removed synonymous sequences through a dereplication step; 4) attributed a code to each retained sequence variant (haplotype) according to the evolutionary lineage they belonged to after manual inspection (i.e. checking GenBank submission metadata) and/or alignment. Starting from 2276 downloaded sequences, we gathered 109 unique variants.

Data analyses

We aligned variants that passed our filtering and assignment criteria, computed nucleotide substitutions between haplotypes and lineages in MEGA 11[51], and explored haplotype dissimilarity by building a Median-Joining network in PopArt[52].

The Spearman rank correlation (*cor.test* R function) was applied to test the association between assigned reads per site (averaged across two replicates) and trout abundance estimates (SC_{2024} and DI_{2024}).

For downstream analyses, conducted in R v.4.4.1[53], we conservatively considered lineages: first, we summed reads by assigned lineage within each of the two replicates (amplicons) per site; then, we computed per site lineage frequencies by averaging relative lineage frequencies obtained from the two field replicates – this was necessary to ensure results were comparable, as amplicon depth may vary among amplicons.

We first assessed the association between raw per-site lineage relative frequencies obtained from eDNA (F_{eDNA}) and tissue samples (F_{tissue}) by computing Pearson correlation and testing its significance (*cor.test* function). We then evaluated consistency between F_{eDNA} and F_{tissue} estimates, while accounting for uncertainty associated with F_{tissue} (e.g., different sample sizes). To do so, we started by fitting a generalized linear mixed model (GLMM) with multinomial error distribution to obtain estimates of F_{tissue} and their associated standard deviations (*SD*). The categorical response in our GLMM was the lineage attributed to each tissue sample, while the sampling site (*site*) was included as a random effect predictor (intercept only). No fixed effect predictors were included in the model. We fitted the GLMM using the *gam* function from package "mgcv" v.1.9.3[54] with the following formula:

$$\text{gam(list(lineage} \sim \text{s(site, bs = "re"), s(site, bs = "re"), s(site, bs = "re"), family = multinom(k = 3)))}$$

The DA lineage, which is entirely absent from tissue samples, was excluded from the possible outcomes and from all further analyses. The predictions from this model for each sampling site (computed using the *predict.gam* function with se.fit = T) are the estimated frequencies (F_{tissue}) and their associated SD_{tissue} for each lineage at each site. By modelling *site* as a random effect, we assumed non-independent lineage frequencies across sampling sites. We considered this assumption reasonable, given the geographic proximity of sampling sites and possibly similar management practices, which we expect to be reflected in loosely related baseline frequencies of lineages. On the other hand, this approach allowed us to obtain non-zero uncertainty estimates (*SD*) for lineage-site combinations with no observations (i.e., when a given lineage was not observed at a given site in the tissue sample), based on the mean frequency of lineages in the overall tissue sample, which would have been impossible if frequencies were assumed to be entirely independent (an arguably even less realistic assumption). We used the *SD* values obtained from the GLMM to compute standardized measures of discordance between F_{eDNA} and F_{tissue} estimates for each combination of lineage and site as $d = (F_{eDNA} - F_{tissue})/SD_{tissue}$. Under the null hypothesis that F_{eDNA} is an infinitely accurate and unbiased ("perfect") estimator of the actual frequency of each lineage at each site from which F_{tissue} were sampled (and assuming that F_{tissue} is an unbiased estimator of actual frequencies), the distribution of d should approximate a normal distribution with $mean = 0$ and $SD = 1$. We tested this null hypothesis by a Kolmogorov-Smirnov test (*ks.test* function). Subsequently, we evaluated how absolute standardized measures of discordance ($|d|$ or *abs_d*) were related to raw values of F_{tissue}, lineage membership and trout density at each sampling site by fitting a generalized additive mixed model (GAMM) with gamma error distribution and inverse link function where *abs_d* was the response, F_{tissue} and the density of trout individuals (DI_{2024}) were smoothed (*thin plate*) fixed effect predictors and lineage membership (*lineage*) was a random effect predictor (intercept only). We also included the sampling site (*site*) as an additional random intercept to account for non-independence of observations within the same sampling site. We used the "mgcv" *gam* function to fit the GAMM model with the formula:

$$\text{gam(abs\_d} \sim \text{s}(F_{tissue}) + \text{s}(DI_{2024}) + \text{s(site, bs = "re")} + \text{s(lineage, bs = "re"))}$$

Note that we initially also considered trout abundances expressed as SC_{2024}, but we excluded it from our final model to avoid collinearity issues, as it showed substantial correlation with DI_{2024} (Pearson $r = 0.72$, $p = 0.02$) and preliminary data explorations indicated DI_{2024} to have a more important effect on *abs_d*.

We compared lineage assemblages as obtained from different genetic sources (eDNA vs. tissue samples) following two approaches based on distance matrices. First, a Mantel test (*mantel* function with 9999 permutations) evaluated the correlation between matrices of Euclidean distances among sites based on F_{eDNA} and F_{tissue}, as computed by the *vegdist* function. Second, a non-metric multidimensional scaling (NMDS) built on a single Euclidean distance matrix was used to visualize lineage assemblages generated by the two methods – we obtained the optimal NMDS configuration by running the *metaMDS* algorithm with 9999 iterations. A permutational analysis of variance (PERMANOVA, *odonis2* function) tested the differences between sites grouped according to genetic source: we expected no statistical differences in case of consistent lineage compositions between eDNA and tissue samples. The above functions were implemented in the "vegan" package[55].

Finally, we evaluated whether the amount of discrepancies in lineage assemblages retrieved by the two methods was affected by environmental or demographic factors. We used the Spearman rank correlation (*cor.test* R function) to test the pairwise association between intra-site Euclidean distance (i.e., the distance computed by comparing eDNA- and tissue-based lineage assemblages) and trout abundance (DI_{2024} and SC_{2024}), site elevation, water flow and water temperature – despite the latter two being single sampling measures, we assumed seasonal fluctuations to vary coherently among sites.

Results

After bioinformatic processing, we obtained on average 223,619 (± 180,584 *SD*) reads per replicate, 5.6% (± 4.9% *SD*) of which remained unassigned according to the adopted criteria (Table S1). We retrieved a total of 12 haplotypes, differing by 1–6 substitutions from each other, and belonging to five mitochondrial lineages (e.g., AD, MA, ME, AT and DA) (Fig. 2, Table S1). Lineage differentiation was maximum between AT and MA (5.33 substitutions on average) and minimum between MA and ME (1.67 substitutions; Table S2). The field blank, the extraction blank and the negative PCR control showed no reads after data processing and haplotype assignment.

Trout abundance estimates, provided in Table 1, were not associated with mean assigned reads per site (Spearman $r = -0.16$ and $p = 0.63$ for SC_{2024}; Spearman $r = 0.12$ and $p = 0.73$ for DI_{2024}).

F_{eDNA} and F_{tissue} were strongly and positively correlated (Pearson $r = 0.93$, $p < 0.0001$). The distribution of standardized discordance measures (d) showed an evident outlier for lineage ME in MOL (Figure S2). In fact, F_{eDNA} (ME) at this site was ~ 17%, while no ME sequence was recovered from tissue DNA (F_{tissue}(ME) = 0). This result is extremely unlikely (> 35 *SD*) if both F_{eDNA}(ME) and F_{tissue}(ME) are unbiased estimators of the same underlying frequency. All other measures of d were between − 7.02 and 4.76 *SD*.

Although the distribution of standardized discordance measures differed significantly from the expectation derived from the null hypothesis of F_{eDNA} being a "perfect" estimator of lineage frequencies (Kolmogorov-Smirnov test, $n = 40$, $D = 0.27$, $p = 0.0035$) it was still relatively close to the latter, with 77.5% of F_{eDNA} estimates within 3 *SDs* of the corresponding F_{tissue} (expected value = 99.8%; Figure S2). The difference remained clearly significant when the outlier F_{eDNA} (ME) was excluded (Kolmogorov-Smirnov test, $n = 39$, $D = 0.26$, $p = 0.0088$), but no more so when all observations where $F_{tissue} = 0$ were excluded (Kolmogorov-Smirnov test, $n = 22$, $D = 0.17$, $p = 0.52$).

Figure 3A, depicting the pattern of $|d|$ with respect to F_{tissue}, shows a cluster of five positive and extreme (> 3 *SD*) values of $|d|$ at $F_{tissue} = 0$. Absolute standardized discordance ($|d|$), however, did not depend on F_{tissue} (GAMM: $F = 0.075$, $p = 0.786$), while our data suggest that it may be related (with marginal significance) to trout density (GAMM: $F = 3.781$, $p = 0.060$; Table 2), with lower discordances associated with higher trout density (Fig. 3B).

Lineage frequencies across sites, depicted in Fig. 4, indicated assemblages' consistency between sources of information (eDNA vs. tissue samples), especially for the within-site most abundant lineages. Matrices of among-site distances (Table S3) were strongly correlated (Mantel $r = 0.834$, $p = 0.0001$).

The NMDS (Fig. 5) returned a highly informative ordination (stress = 3.9%), clearly separating samples according to the lineage composition, i.e., assemblages dominated by the AD, AT or ME lineage. Samples from the same site but different sources were mostly located nearby. Convex hulls including eDNA and tissue-based samples largely overlapped – the PERMANOVA test found no statistical differences between groups, indeed ($F_{1,19} = 0.09$, $p = 0.940$).

Intra-site Euclidean distance was negatively correlated with DI_{2024} (Spearman $r = -0.644$, $p = 0.044$) and positively correlated with water temperature (Spearman $r = 0.723$, $p = 0.018$), while no significant rank association emerged with other environmental/demographic parameters tested.

Discussion

In this study, we developed and validated a straightforward, reliable, and non-invasive method to reconstruct evolutionary mitochondrial DNA lineage assemblages in wild brown trout populations through next-generation amplicon sequencing of eDNA filtered from small volumes of stream water.

We found no (rank) association between electrofishing-estimated trout abundance in wild populations and amplicon sequencing-generated read counts, suggesting the latter as a non-accurate proxy of fish eDNA concentration in examined watercourses. This apparently contrasts with results from other studies targeting eDNA of wild Salmonids, while employing next-generation amplicon sequencing:[35] found positive relationships of various strengths between brown trout density/biomass in Spanish mountain watercourses and eDNA concentration obtained by species-specific quantitative PCR or eDNA abundance approximated by logarithmic-transformed read counts from metabarcoding;[56] revealed a relationship between normalized eDNA read counts and biomasses of *Salmo salar* in the River Conway (United Kingdom), which interestingly linearized at > 1500 kg fish. However, multiple concurring biotic and abiotic factors are known to introduce uncertainty in absolute estimates of eDNA abundance[8], possibly affecting the overall eDNA-trout abundance relationship

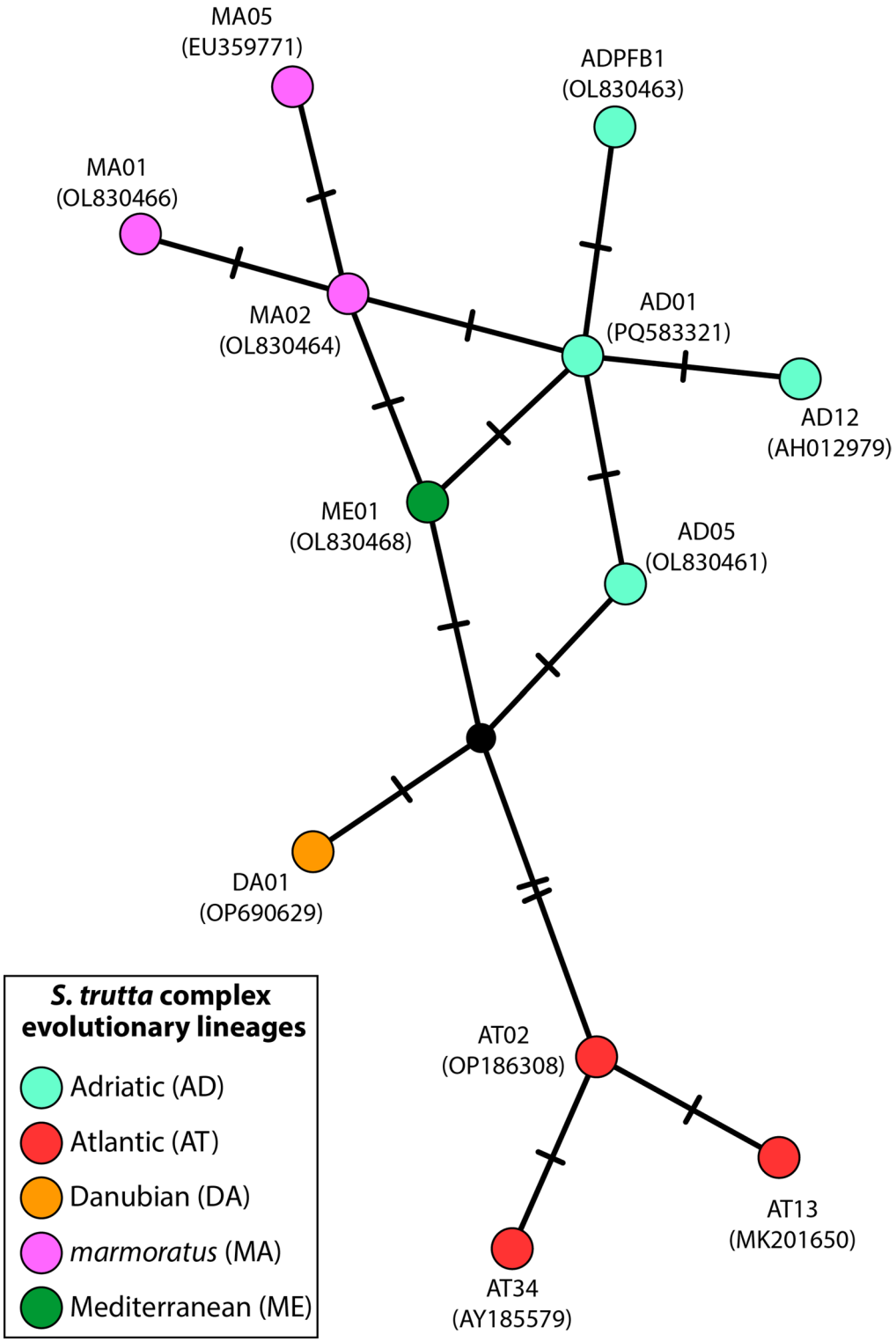

Fig. 2. Median-Joining network of 12 mitochondrial Control Region variants (haplotypes) of 192 bp found in 10 brown trout wild sites after the analysis of eDNA water samples. Labels indicate haplotype names and GenBank codes (in parentheses); colours distinguish among evolutionary lineages; bars indicate the number of substitutions between haplotypes.

across ecologically heterogeneous watercourses sampled in this study. Besides individual variation in eDNA shedding rates[57] and PCR stochasticity/amplification biases[58,59], differences in water temperature[60] and flow[56] may also have contributed. Furthermore, spatial-temporal movement/dispersal of eDNA can considerably vary across sites – eDNA molecules can persist for days/weeks[61] while transported downstream by drift, even for

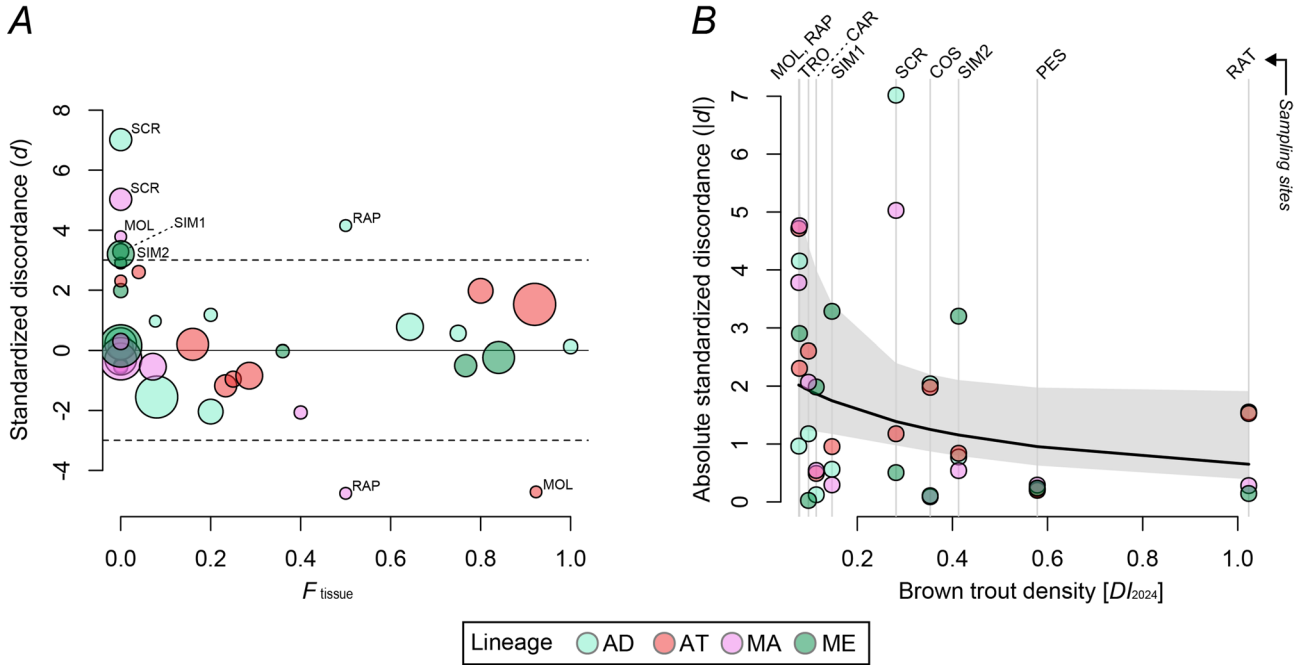

Fig. 3. Patterns of standardized discordance. (**A**) Relationship between standardised discordance (d) and per-site lineage frequency as estimated from tissue samples (F_{tissue}); dot areas are proportional to per-site tissue sample sizes (as in Table 1); for a better visualization, only lineage-site combinations outside the range $d = \pm 3$ *SD* (dashed lines) are labelled. (**B**) Relationship between trout density (DI_{2024}) and absolute standardized discordance ($|d|$); vertical solid lines connect measures from the same sampling site; the solid black line is the fit from the GAMM with 95% confidence intervals (grey shaded area). Dot colours indicate lineages in both figures.

Predictor	Estimate df	Reference df	F	*p*
F_{tissue}	1.000	1	0.075	0.786
DI_{2024}	1.000	1	3.781	0.060
site (Random effect)	1.223	8	0.238	0.181
lineage (Random effect)	0.00002	3	0.000	0.958

Table 2. Results of GAMM assessing the effect of per-site lineage frequency based on tissues (F_{tissue}) and trout abundance (expressed as density index = DI_{2024}) on standardized absolute discordances ($|d|$), with sampling site (Site) and lineage membership (Lineage) as controlling random effects. For each predictor/random effect, the estimate/reference degrees of freedom (df) are provided along with the *F*-statistic and the corresponding *p*-value. The model explains an overall 17% deviance.

kilometres[33,62] – leading to inconsistency with fish abundance estimates based on electrofishing along small river stretches.

Major results from this study highlight the effectiveness of eDNA in accurately reconstructing brown trout population diversity based on the relative frequencies of (co)occurring evolutionary mitochondrial lineages. Indeed, the fundamental genetic structure of the examined populations, characterized by different within-site dominant lineage(s), appears highly preserved by eDNA sampling, as indicated by the considerable agreement between (semi-quantitative) lineage assemblages returned by traditional genotyping and eDNA analysis (Mantel test, NMDS, and PERMANOVA). Furthermore, per-site lineage frequencies estimated by the two methods were strongly correlated (Pearson r = 0.93), an outcome even more impressive considering that the timespan between tissue and eDNA sampling (5–6 years) may imply an underestimation of accordance. This crucial result corroborates with a few previous studies targeting marine taxa, the Ayu and a whale shark, where within-population haplotype frequencies, obtained with different eDNA typing techniques, robustly reflected those obtained via traditional genotyping[63,64].

Counterintuitively, we found that eDNA behaves as a statistically "non-perfect" estimator of the actual lineage frequencies. However, a fraction of relatively large discordances estimated for lineages non-observed in the tissue samples (Fig. 3A) has likely driven the significance of tests (Kolmogorov-Smirnov results before vs. after removing $F_{tissue} = 0$ occurrences). Analogously to metabarcoding studies (e.g[6]), the detection of previously unrecorded lineages at low frequencies across sampling sites may indicate enhanced power of eDNA to detect rare

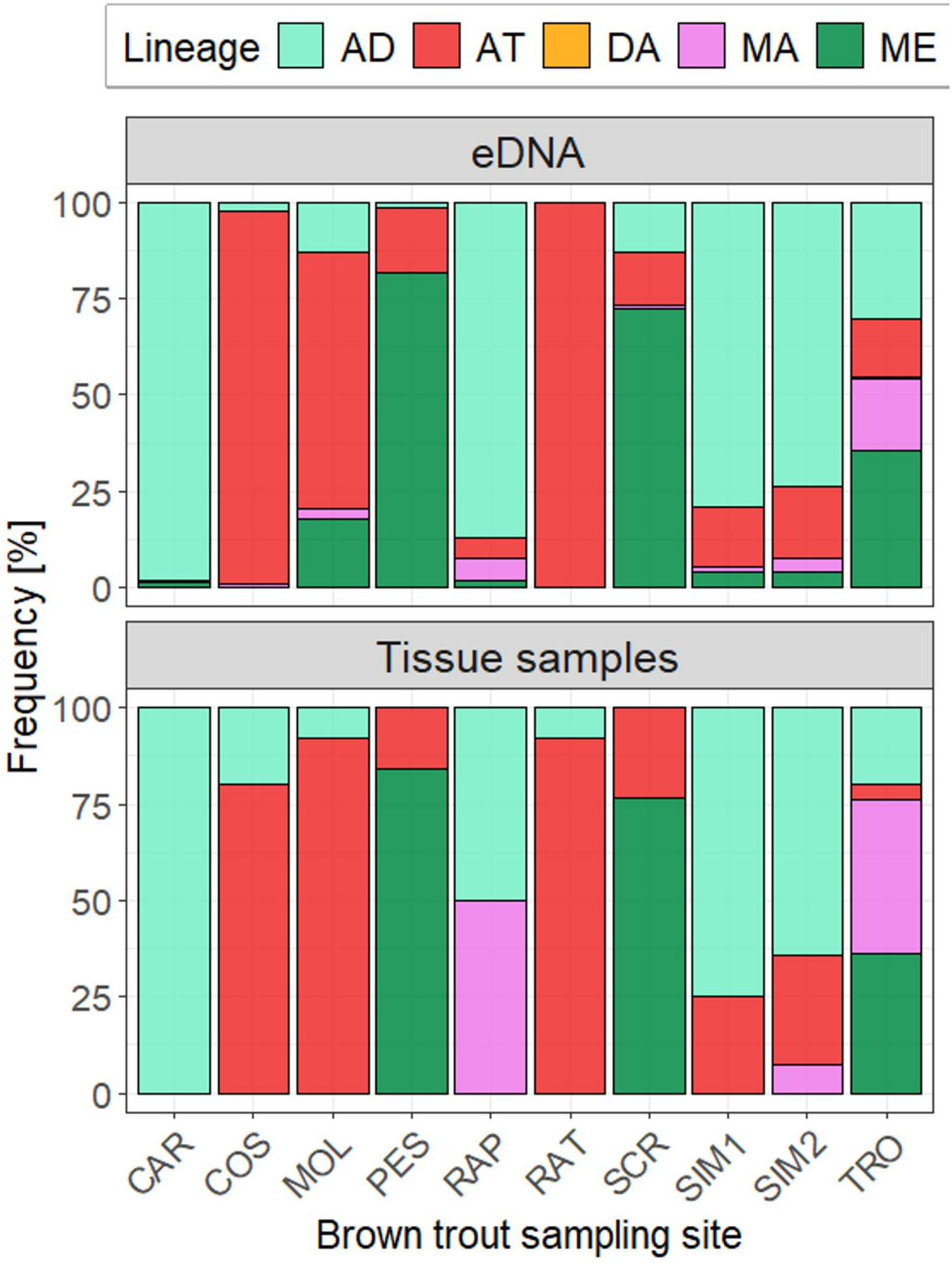

Fig. 4. Bar charts indicating relative frequencies of brown trout mitochondrial Control Region lineages detected in 10 sampling sites by next-generation amplicon sequencing of eDNA from filtered stream water (above) and traditional genotyping of tissue samples (below). Note that the DA lineage occurs in the eDNA TRO sample at 0.2% frequency.

lineages over a potentially much more extended upstream sampling area, including electrofishing-unsampleable river stretches. Of course, this may also indicate false positives originating from contamination during sampling and laboratory procedures[65]. To take into account these, we collected a negative control for each major step of eDNA processing (fieldwork, DNA extraction and PCR amplification), but it is fair to note that collecting

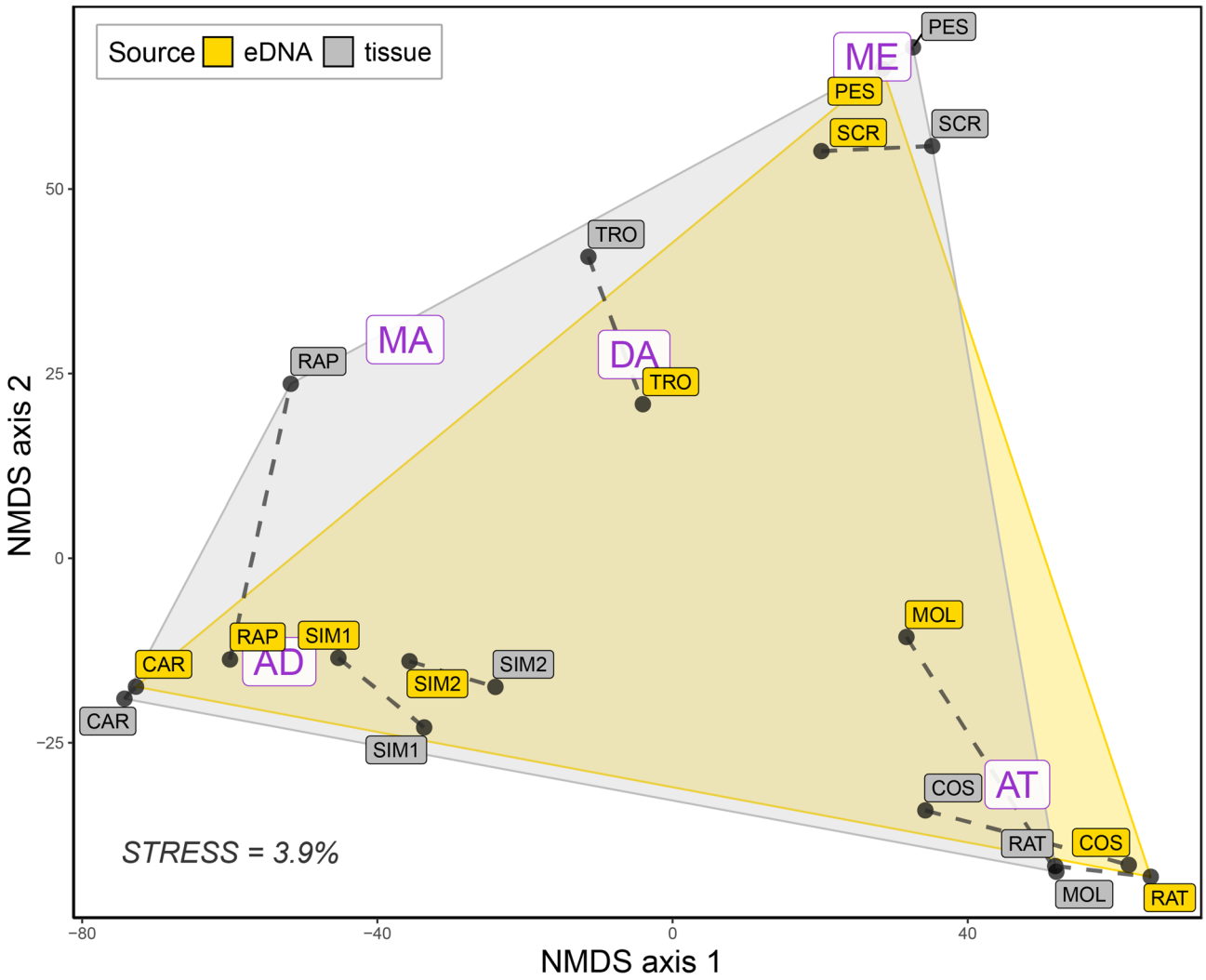

Fig. 5. Non-metric two-dimensional scaling of Euclidean distances among sites based on brown trout lineage assemblages retrieved from eDNA and tissue samples. Site codes refer to Table 1, and labels are coloured according to source of genetic information – dotted segments join observation pairs from the same sampling sites.

multiple field negative controls would have improved estimates of almost unavoidable contaminations associated with eDNA sampling. Among other sources of false positives, we considered the effect of tag jumping negligible because of the lower rate reported for the DNBSEQ-G400 compared to other next-generation-sequencing platforms[66] and the adopted dual unique tagging approach[1]. Analogously, occurring PCR/sequencing artefacts[15] should result in limited changes, as we collapsed haplotypes into (well)differentiated mitochondrial lineages, an operational procedure that desirably reduces effort/optimization in data cleaning and variant assignment to ultimately distinguish between putatively true haplotypes and artefacts[10,67]. On the other hand, such a conservative procedure, coupled with the amplification of a shortened CR fragment, obviously results in an underestimation of population mitochondrial diversity – an acceptable bias when eDNA is primarily intended as a non-invasive monitoring tool for preliminary characterization of trout diversity in wild populations. Among eDNA-exclusive occurrences, the DA lineage found in TRO (Tronto River, flowing into the central Adriatic Sea), even if at very low frequency in a single replicate (Table S1), remains particularly controversial and would deserve further investigations to clarify its actual presence and natural/anthropogenic origin. Indeed, the DA lineage is widespread in Balkan catchments[17], while it has been recorded only in subalpine watercourses in Italy – to the best of our knowledge, in a few tributaries of the Danubian/Rhine drainages (e.g., Slizza, Inn, and Drave) and South Tyrol watercourses, even though the origin of populations in the latter remains unclear[68,69].

Besides possible false positives or previously undetected true (rare) lineages, observed discordances should be interpreted as temporal (natural) fluctuations in lineage frequencies within sites occurred in the 5–6 year timespan between tissue and eDNA sampling (e.g., AD and MA in RAP, AT in MOL), rather than uncertainty linked to tissue-based estimates (non-significance of F_{tissue} in GAMM). However, we cannot rule out the hypothesis that strong deviations from expected frequencies (i.e., very large standard discordances) may result from impactful events, such as human-mediated stocking – this could be the case of the ME "outlier" in MOL,

as unusual stocking/translocations of trout carrying native lineages have been recorded in Italy[25,41,70]. From a conservation perspective, our data generally indicate that no or minimal manipulations have occurred in examined populations in recent years (no evident increases of the hatchery AT lineage across sites), likely because of the almost concomitant SARS-CoV-19 pandemic[71] and the coming into effect of an Italian regulation *de facto* suspending stocking activities with exotic domestic Atlantic strains since 2020[72].

Finally, we interestingly found that increasing trout density and water temperature, respectively, improved and reduced concordance between semi-quantitative lineage assemblages inferred by eDNA and traditional genotyping (output from GAMM and Spearman correlations). Both associations could be explained in the light of actual amounts of relatively well-preserved eDNA in the watercourses, as higher eDNA concentrations intuitively result in more accurate estimates of lineage frequency, particularly by reducing the risk of haplotype dropout due to low-abundant or fragmented DNA[73,74]. Anyhow, while a positive relationship between eDNA concentration and fish abundance is commonly reported even in natural systems[8], the effect of temperature remains controversial. On one hand, higher water temperatures may accelerate eDNA degradation because of enhanced microbial metabolism and enzyme activity, consequently reducing available DNA of sufficient quality for PCR amplification[75]. On the other hand[60], experimentally demonstrated that *Salverinus fontalis* shed more eDNA in warm than in cold water, likely due to augmented fish activity and metabolism, eventually showing that eDNA concentration better mirrored fish abundance/biomass at higher temperatures.

Conclusions and perspectives

Overall, this study has contributed to the understanding of the effectiveness of eDNA for investigating intraspecific genetic diversity in aquatic taxa. In the case of brown trout, lineage diversity can inform the genetic conservation status of wild populations in the Mediterranean region. Therefore, our eDNA protocol may serve as a valuable tool to support monitoring efforts outlined by international (e.g., the European Habitat Directive and the Water Framework Directive) or local legislation. For endangered taxa within the *S. trutta* complex (e.g., *Salmo ghigii*, classified as Endangered by IUCN[76]), dedicated conservation projects might benefit from such rapid screening or assessment, for example, to facilitate the identification of suitable populations for spawner selection in genetic enhancement programmes[28], or to reveal residual native populations in suitable but poorly explored areas, such as lowland cold-water refugia[77].

At last, although specifically not assessed in this study, experimental and in silico cross-amplification tests indicated successful amplification of the target CR fragments in other Salmonids that can co-occur in brown trout wild sites, either as sympatric native or stocked/escaped alien species of economic interest (e.g. *Oncorhynchus spp*., *Salmo salar*, *Salvelinus spp.*[78]). As further evidence, a manual (BLAST) check of unassigned reads in our eDNA samples revealed some sequences matching with *O. mykiss* and *Salvelinus sp*. in TRO and SIM2, and, importantly, both taxa were confirmed by contextual electrofishing sampling in SIM2.

This paves the way for future investigations to evaluate the effectiveness of our primer pair for eDNA-based biomonitoring and intraspecific mitochondrial diversity assessments across multiple Salmonids simultaneously. Experiments in controlled but realistic (semi-natural) conditions would represent an important next step to assess the robustness and applicability of this approach.

Data availability

Amplicon sequencing raw output (two paired-end FastQ files) is available on NCBI as BioProject no. PRJNA1328505, BioSample no. SAMN51337112, and Sequence Read Archive (SRA) no. SRR35396690.

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References

1. Taberlet, P., Bonin, A., Zinger, L. & Coissac, E. *Environmental DNA: For Biodiversity Research and Monitoring* (Oxford University Press, 2018).
2. Yao, M. et al. Fishing for fish environmental DNA: Ecological applications, methodological considerations, surveying designs, and ways forward. *Mol. Ecol.* **31**, 5132–5164 (2022).
3. Bohmann, K. et al. Environmental DNA for wildlife biology and biodiversity monitoring. *Trends Ecol. Evol.* **29**, 358–367 (2014).
4. Wang, S. et al. Methodology of fish eDNA and its applications in ecology and environment. *Sci. Total Environ.* **755**, 142622 (2021).
5. Milhau, T. et al. Seasonal dynamics of riverine fish communities using eDNA. *J. Fish Biol.* **98**, 387–398 (2021).
6. Maiello, G. et al. Fishing in the gene-pool: Implementing trawl-associated eDNA metaprobe for large scale monitoring of fish assemblages. *Rev. Fish. Biol. Fish.* **34**, 1293–1307 (2024).
7. Tetzlaff, S., Katz, A., Johnson, M. & Sperry, J. Community ecology in a bottle: Leveraging eDNA metabarcoding data to predict occupancy of co-occurring species. *Environ. DNA* **6**, e579 (2024).
8. Rourke, M. L. et al. Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. *Environ. DNA* **4**, 9–33 (2022).
9. Adams, C. I. M. et al. Beyond biodiversity: Can environmental DNA (eDNA) cut it as a population genetics tool? *Genes* **10**, 192 (2019).
10. Weitemier, K. et al. Estimating the genetic diversity of Pacific Salmon and trout using multigene eDNA metabarcoding. *Mol. Ecol.* **30**, 4970–4990 (2021).
11. Andres, K. J., Lodge, D. M., Sethi, S. A. & Andrés, J. Detecting and analysing intraspecific genetic variation with eDNA: From population genetics to species abundance. *Mol. Ecol.* **32**, 4118–4132 (2023).
12. Wakimura, K., Yonekura, R., Yamanaka, H. & Uchii, K. Environmental DNA haplotyping reveals dispersal patterns of invasive Bluegill Sunfish, *Lepomis macrochirus*, in Japan. *Environ. DNA* **7**, e70055 (2025).
13. Halvorsen, S., Korslund, L., Mattingsdal, M. & Slettan, A. Estimating number of European eel (*Anguilla anguilla*) individuals using environmental DNA and haplotype count in small rivers. *Ecol. Evol.* **13**, e9785 (2023).
14. Dugal, L. et al. Individual haplotyping of Whale sharks from seawater environmental DNA. *Mol. Ecol. Resour.* **22**, 56–65 (2022).

15. Sigsgaard, E. E. et al. Population-level inferences from environmental DNA—Current status and future perspectives. *Evol. Appl.* **13**, 245–262 (2020).
16. Deiner, K. et al. Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Mol. Ecol.* **26**, 5872–5895 (2017).
17. Tougard, C. Will the genomics revolution finally solve the *Salmo* systematics ? *Hydrobiologia* **849**, 2209–2224 (2022).
18. Bernatchez, L. The evolutionary history of brown trout (*Salmo trutta* L.) inferred from phylogeographic, nested clade, and mismatch analyses of mitochondrial DNA variation. *Evolution* **55**, 351–379 (2001).
19. Sanz, N. Phylogeographic history of brown trout: A review. in Brown Trout: Biology, Ecology and Management (eds Lobón-Cerviá, J. & Sanz, N.) 2–63 (John Wiley & Sons, Ltd, 2018). https://doi.org/10.1002/9781119268352.ch3.
20. Veličković, T. et al. A new perspective on the molecular dating of the brown trout complex with an extended phylogeographic information on the species in Serbia. *Contrib. Zool.* **92**, 362–389 (2023).
21. MacCrimmon, H. R. & Marshall, T. L. World distribution of brown Trout, *Salmo trutta*. *J. Fish. Res. Board Can.* **25**, 2527–2548 (1968).
22. Pinter, K., Epifanio, J. & Unfer, G. Release of hatchery-reared brown trout (*Salmo trutta*) as a threat to wild populations? A case study from Austria. *Fish. Res.* **219**, 105296 (2019).
23. Berrebi, P., Horvath, Á., Splendiani, A., Palm, S. & Bernaś, R. Genetic diversity of domestic brown trout stocks in Europe. *Aquaculture* **544**, 737043 (2021).
24. Berrebi, P. et al. Brown trout (*Salmo trutta* L.) high genetic diversity around the tyrrhenian sea as revealed by nuclear and mitochondrial markers. *Hydrobiologia* **826**, 209–231 (2019).
25. Rossi, A. R. et al. Genetic identification of native populations of mediterranean brown trout *Salmo trutta* L. complex (Osteichthyes: Salmonidae) in central Italy. *Eur. Zoological J.* **86**, 424–431 (2019).
26. Splendiani, A. et al. Introgression despite protection: The case of native brown trout in natura 2000 network in Italy. *Conserv. Genet.* **20**, 343–356 (2019).
27. Vera, M. et al. Identification of an endemic mediterranean brown trout MtDNA group within a highly perturbed aquatic system, the Llobregat river (NE Spain). *Hydrobiologia* **827**, 277–291 (2019).
28. Carosi, A. et al. The LIFE STREAMS project for the recovery of the native mediterranean trout in six Italian pilot areas: Planning and adoption of conservation actions. *Biology* **14**, 573 (2025).
29. Banks, J. C., Demetras, N. J., Hogg, I. D. & West, D. W. Monitoring brown trout (*Salmo trutta*) eradication in a wildlife sanctuary using environmental DNA. *New. Zeland Nat. Sci.* **41**, 1–13 (2016).
30. Carim, K. J. et al. An environmental DNA marker for detecting nonnative brown trout (*Salmo trutta*). *Conserv. Genet. Resour.* **8**, 259–261 (2016).
31. Kim, S. H., Lee, S. I., Lee, S. H., Jo, S. E. & Kim, K. Y. The molecular monitoring of an invasive freshwater Fish, brown trout (*Salmo trutta*), using Real-Time PCR assay and environmental water samples. *Animals* **15**, 659 (2025).
32. 100 of the World's. Worst invasive alien species: A selection from the global invasive species database. In *The Invasive 1518 Species Specialist Group (ISSG) a Specialist Group of the Species Survival Commission 1519 (SSC) of the World Conservation Union (IUCN)*. (eds Lowe, S., Browne, M. & Boudjelas, S.) vol. 12 1–12 (University of California Press, 2000).
33. Deutschmann, B., Müller, A. K., Hollert, H. & Brinkmann, M. Assessing the fate of brown trout (*Salmo trutta*) environmental DNA in a natural stream using a sensitive and specific dual-labelled probe. *Sci. Total Environ.* **655**, 321–327 (2019).
34. Capo, E., Spong, G., Königsson, H. & Byström, P. Effects of filtration methods and water volume on the quantification of brown trout (*Salmo trutta*) and Arctic Char (*Salvelinus alpinus*) eDNA concentrations via droplet digital PCR. *Environ. DNA* **2**, 152–160 (2020).
35. Losa, A. M. et al. Metabarcoding versus Species-Specific primers to estimate *Salmo trutta* biomass and density in mountain streams. *Environ. DNA*. **7**, e70090 (2025).
36. Rossi, A. R., Talarico, L., Petrosino, G., Crescenzo, S. & Tancioni, L. Conservation genetics of mediterranean brown trout in central Italy (Latium): A multi-marker approach. *Water* **14**, 937 (2022).
37. Moran, P. A. P. A mathematical theory of animal trapping. *Biometrika* **38**, 307–311 (1951).
38. Zippin, C. An evaluation of the removal method of estimating animal populations. *Biometrics* **12**, 163 (1956).
39. Palombo, V. et al. Genotyping of two Mediterranean trout populations in central-southern Italy for conservation purposes using a rainbow-trout-derived SNP array. *Animals* **11**, 1803 (2021).
40. Splendiani, A. et al. Population genetics, demography and conservation of Mediterranean brown trout from Sardinia. *Aquat. Conserv. Mar. Freshw. Ecosyst.* **2**, e4099 (2024).
41. Talarico, L. et al. Population structure, genetic diversity and demographic patterns unveil massive mediterranean brown trout manipulations in a protected area of the Northern apennines (Italy). *Eur. Zool. J.* **90**, 470–486 (2023).
42. Untergasser, A. et al. Primer3—New capabilities and interfaces. *Nucleic Acids Res.* **40**, e115–e115 (2012).
43. Sabatini, A. et al. Genetic characterization and artificial reproduction attempt of endemic Sardinian trout *Salmo trutta* L., 1758 (Osteichthyes, Salmonidae): Experiences in captivity. *Italian J. Zool.* **78**, 20–26 (2011).
44. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).
45. Schnell, I. B., Bohmann, K. & Gilbert, M. T. P. Tag jumps illuminated—reducing sequence-to-sample misidentifications in metabarcoding studies. *Mol. Ecol. Resour.* **15**, 1289–1303 (2015).
46. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **17**, 10–12 (2011).
47. Bolyen, E. et al. Reproducible, interactive, scalable and extensible Microbiome data science using QIIME 2. *Nat. Biotechnol.* **37**, 852–857 (2019).
48. Callahan, B. J. et al. DADA2: High-resolution sample inference from illumina amplicon data. *Nat. Methods* **13**, 581–583 (2016).
49. Bokulich, N. A. et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* **6**, 90 (2018).
50. Jeunen, G. et al. Crabs—A software program to generate curated reference databases for metabarcoding sequencing data. *Mol. Ecol. Resour.* **23**, 725–738 (2023).
51. Tamura, K., Stecher, G. & Kumar, S. MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* **38**, 3022–3027 (2021).
52. Leigh, J. & Bryant, D. PopART: Full-feature software for haplotype network construction. *Methods Ecol. Evol.* **6**, 1110–1116 (2015).
53. R Core Team. *R: A Language and Environment for Statistical Computing* (R Foundation for Statistical Computing, 2024).
54. Wood, S. N. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. *J. Royal Stat. Soc. Ser. B: Stat. Methodol.* **73**, 3–36 (2011).
55. Oksanen, J. et al. vegan: Community Ecology Package (2025).
56. B Perry, W. et al. Quantitative insights into the spatio-temporal variation of Atlantic salmon (Salmo salar) biomass in a river catchment using eDNA metabarcoding. *J. Fish Biol.* (in press). https://doi.org/10.1111/jfb.70172 (2025).
57. Klymus, K. E., Richter, C. A., Chapman, D. C. & Paukert, C. Quantification of eDNA shedding rates from invasive Bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biol. Conserv.* **183**, 77–84 (2015).
58. Elbrecht, V. & Leese, F. Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass—sequence relationships with an innovative metabarcoding protocol. *PloS One.* **10**, e0130324 (2015).
59. Shelton, A. O. et al. Toward quantitative metabarcoding. *Ecology* **104**, e3906 (2023).

60. Lacoursière-Roussel, A., Rosabal, M. & Bernatchez, L. Estimating fish abundance and biomass from eDNA concentrations: Variability among capture methods and environmental conditions. *Mol. Ecol. Resour.* **16**, 1401–1414 (2016).
61. Dejean, T. et al. Persistence of environmental DNA in freshwater ecosystems. *PLoS ONE* **6**, e23398 (2011).
62. Pont, D. et al. Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. *Sci. Rep.* **8**, 1–13 (2018).
63. Sigsgaard, E. E. et al. Population characteristics of a large Whale shark aggregation inferred from seawater environmental DNA. *Nat. Ecol. Evol.* **1**, 0004 (2016).
64. Tsuji, S., Shibata, N., Sawada, H. & Ushio, M. Quantitative evaluation of intraspecific genetic diversity in a natural fish population using environmental DNA analysis. *Mol. Ecol. Resour.* **20**, 1323–1332 (2020).
65. Mathieu, C. et al. A systematic review of sources of variability and uncertainty in eDNA data for environmental monitoring. *Front. Ecol. Evol.* **8**, 135 (2020).
66. Jia, Y. et al. Sequencing introduced false positive rare taxa lead to biased microbial community diversity, assembly, and interaction interpretation in amplicon studies. *Environ. Microbiome.* **17**, 43 (2022).
67. Turon, X., Antich, A., Palacín, C., Præbel, K. & Wangensteen, O. S. From metabarcoding to metaphylogeography: Separating the wheat from the chaff. *Ecol. Appl.* **30**, e02036 (2020).
68. Meraner, A., Baric, S., Pelster, B. & Dalla Via, J. Trout (*Salmo trutta*) mitochondrial DNA polymorphism in the centre of the marble trout distribution area. *Hydrobiologia* **579**, 337–349 (2007).
69. Pujolar, J. M. et al. The effect of recurrent floods on genetic composition of marble trout populations. *PLoS ONE*. **6**, e23822 (2011).
70. Fabiani, A. et al. Investigating the genetic structure of trout from the garden of Ninfa (central Italy): Suggestions for conservation and management. *Fish. Manag. Ecol.* **25**, 1–11 (2018).
71. Cooke, S. J. et al. A global perspective on the influence of the COVID-19 pandemic on freshwater fish biodiversity. *Biol. Conserv.* **253**, 108932 (2021).
72. Italy. Ministerial Decree 2 April 2020 on criteria for the reintroduction and repopulation of native species as per Annex D of the Decree of the President of the Republic 8 September 1997, n. 357, and for the introduction of non-native species and populations (Gazzetta Ufficiale della Repubblica Italiana No. 98 of 14 April 2020) (20A02112). (2020).
73. Smith, O. & Wang, J. When can noninvasive samples provide sufficient information in conservation genetics studies? *Mol. Ecol. Resour.* **14**, 1011–1023 (2014).
74. Van Driessche, C., Everts, T., Neyrinck, S. & Brys, R. Experimental assessment of downstream environmental DNA patterns under variable fish biomass and river discharge rates. *Environ. DNA* **5**, 102–116 (2023).
75. Kasai, A., Takada, S., Yamazaki, A., Masuda, R. & Yamanaka, H. The effect of temperature on environmental DNA degradation of Japanese eel. *Fish. Sci.* **86**, 465–471 (2020).
76. Ford, M. *Salmo ghigii*. *The IUCN Red List of Threatened Species 2025* e.T274318228A274319393 (2025). https://doi.org/10.2305/IUCN.UK.2025-1.RLTS.T274318228A274319393.en
77. Talarico, L., Catucci, E., Martinoli, M., Scardi, M. & Tancioni, L. Modelling *Salmo trutta* complex Spatial distribution in central italy: A random forest approach revealing underrepresented lowland populations based on spatially-explicit predictors. *Ecol. Evol.* **15**, e71658 (2025).
78. McMillan, J. R. et al. A global synthesis of peer-reviewed research on the effects of hatchery salmonids on wild salmonids. *Fish. Manag. Eco.* **30**, 446–463 (2023).

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Author contributions

Conceptualization: LTan, LTal, AR; Formal Analyses: LTal, PG; Investigations: GP, LTal, LTan; Methodology: PG, LTal; Resources: PF, LTan, AR; Visualization: LTal, PG; Writing - Original Draft: LTal, PG, GP; Writing - Review & Editing: LTal, GP, AR, LTan, PF, PG.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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