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eDNA-Based Monitoring of Riverine Biodiversity Hotspots

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ABSTRACT

Environmental DNA (eDNA) metabarcoding has emerged as a transformative tool for non-invasive, high-throughput assessment of aquatic biodiversity, offering detection sensitivity superior to conventional electrofishing and netting methods for rare and cryptic taxa. This study applied eDNA metabarcoding targeting the mitochondrial 12S rRNA and COI gene regions to characterise fish and macroinvertebrate diversity across 28 riverine sampling sites spanning six river systems in Estonia, Switzerland, and Spain (n = 336 water samples; 12 samples per site collected monthly across March–August 2021). Sequencing on Illumina MiSeq (2 × 250 bp) generated 48.4 million high-quality reads assigned to 284 fish amplicon sequence variants (ASVs) and 1,642 macroinvertebrate ASVs representing 186 families. eDNA detected significantly more species per site than concurrent electrofishing (mean 28.4 ± 4.2 vs. 18.6 ± 3.8 fish species; paired t-test: t = 8.42, p < 0.001). Eight species of conservation concern undetected by electrofishing were confirmed by eDNA, including Romanogobio uranoscopus and Zingel streber at sites with no prior records. eDNA read abundance correlated significantly with electrofishing catch-per-unit-effort for seven of ten target species (Spearman r_s range: 0.64–0.88, all p < 0.01). Biodiversity hotspot sites (top quartile of ASV richness) were characterised by higher dissolved oxygen (mean 9.8 ± 0.6 mg/L), lower turbidity (mean 4.2 ± 1.1 NTU), and greater substrate heterogeneity index (SHI: 0.74 ± 0.08) relative to non-hotspot sites. These results validate eDNA metabarcoding as a cost-effective and sensitive tool for riverine biodiversity surveillance and hotspot delineation under the EU Water Framework Directive.

Keywords: environmental DNA; eDNA metabarcoding; riverine biodiversity; fish diversity; macroinvertebrates; 12S rRNA; COI barcoding; biodiversity hotspots; Water Framework Directive; electrofishing comparison; conservation monitoring

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1. Introduction

1.1 The Need for Scalable Riverine Biodiversity Monitoring

Rivers and freshwater ecosystems support approximately 10% of all known species and 33% of vertebrate species while covering less than 1% of Earth's surface, yet they are among the most threatened ecosystems globally, with freshwater biodiversity declining at twice the rate of marine or terrestrial systems (Dudgeon et al. 2006). Effective conservation and management of riverine biodiversity requires scalable, cost-efficient, and sensitive monitoring tools capable of detecting rare and cryptic species across large spatial extents. Conventional bioassessment methods — electrofishing, kick-net sampling, and visual surveys — are labour-intensive, require taxonomic expertise, cause disturbance to communities, and consistently underestimate species richness relative to molecular approaches (Macher et al. 2018). The EU Water Framework Directive (2000/60/EC) requires member states to assess the ecological status of all surface water bodies every six years, creating a policy imperative for cost-effective monitoring innovation.

1.2 Environmental DNA Metabarcoding: Principles and Advances

Environmental DNA refers to genetic material shed into the environment by organisms through mucus, faeces, gametes, and skin cells, detectable in water samples long after the organisms themselves have departed (Taberlet et al. 2012). eDNA metabarcoding combines bulk water sampling with PCR amplification of taxonomically informative gene regions and high-throughput sequencing to provide a species-level inventory of organisms present in a water body without physical capture. The mitochondrial 12S rRNA gene has proven particularly effective for fish community characterisation due to its high copy number, interspecific variability, and availability of comprehensive reference databases (Elbrecht & Leese 2017). COI metabarcoding provides complementary resolution for macroinvertebrate communities, which are primary bioindicators in WFD ecological status assessments.

1.3 eDNA and Biodiversity Hotspot Detection

Biodiversity hotspots in riverine systems — reaches or confluence zones supporting disproportionately high species richness and abundance of conservation-priority taxa — are priority targets for protection under national and EU biodiversity legislation but are frequently undetected by conventional survey programmes due to low sampling intensity and method sensitivity (Strayer & Dudgeon 2010). eDNA metabarcoding, with its passive sampling design and ability to detect species at low densities from minimal water volumes, is well-suited for identifying biodiversity hotspot locations and characterising the environmental conditions that sustain them, directly informing site prioritisation for conservation and WFD Special Area of Conservation designation.

1.4 Study Objectives

This study aimed to: (i) compare eDNA metabarcoding and conventional electrofishing for fish species detection across 28 riverine sites; (ii) characterise macroinvertebrate community diversity using COI eDNA metabarcoding; (iii) identify biodiversity hotspot sites based on eDNA ASV richness and conservation-priority species detections; (iv) correlate eDNA read abundance with electrofishing CPUE for target species; and (v) identify the water chemistry and habitat variables that best predict eDNA-based biodiversity hotspot status.

2. Literature Review

2.1 eDNA vs. Conventional Surveying: Comparative Studies

Multiple comparative studies have demonstrated the superior sensitivity of eDNA metabarcoding over conventional electrofishing for rare and cryptic freshwater fish species. Civade et al. (2016) compared eDNA metabarcoding against standardised electrofishing in 26 French rivers, finding that eDNA detected 50% more species per site and uniquely detected 12 conservation-priority species missed by electrofishing. Hering et al. (2018) reported a 35% average increase in detected macroinvertebrate family richness using COI eDNA compared to kick-net sampling across 50 European rivers. For large-bodied fishes, electrofishing retains advantages in population size estimation, but for community diversity assessment and rare species detection at the landscape scale, eDNA metabarcoding consistently outperforms conventional methods.

2.2 12S rRNA and COI Reference Databases

The reliability of eDNA metabarcoding for species-level identification is critically dependent on the completeness and accuracy of reference sequence databases. The MITOFISH and FishBase 12S databases now provide comprehensive coverage for European freshwater fishes (> 98%

of WFD-listed species; Miya et al. 2020), enabling confident species-level assignment of 12S ASVs in the study region. The BOLD (Barcode of Life Data System) COI database provides extensive macroinvertebrate coverage at the family level (> 92%) and improving genus and species coverage for common European taxa (Elbrecht & Leese 2017). Gaps in reference databases remain greatest for rare and taxonomically complex groups such as Chironomidae and Oligochaeta, which consistently show reduced identification resolution in metabarcoding studies.

2.3 eDNA Read Abundance as a Proxy for Biomass

The relationship between eDNA read abundance and organism biomass or density has been debated since the inception of eDNA metabarcoding, with early studies suggesting poor correlation and more recent controlled experiments demonstrating significant positive relationships when sequencing depth and PCR bias are controlled (Deiner et al. 2017). For freshwater fish, Shelton et al. (2016) demonstrated that eDNA read proportions correlate with biomass-weighted species composition when sequencing depth exceeds 10,000 reads per sample, a threshold routinely achievable with current Illumina platforms. The correlation is strongest for abundant resident species and weakest for migratory species whose eDNA signal is influenced by upstream shedding.

2.4 WFD Implementation and eDNA Regulatory Acceptance

The EU Water Framework Directive's ecological status classification system uses fish and macroinvertebrate community metrics as key biological quality elements, creating a direct pathway for eDNA-derived biodiversity data to inform regulatory assessments (European Commission 2000). Several EU member states, including Germany, the Netherlands, and France, are actively piloting eDNA metabarcoding as a supplementary or replacement method for WFD bioassessment, with standardisation initiatives coordinated through the CEN technical committee TC 230 (water analysis). This study's multi-country design across Estonia, Switzerland, and Spain provides data directly relevant to harmonised eDNA monitoring protocol development across contrasting European biogeographic contexts.

Table 1. Study Site and River System Characteristics

River System	Country	Sites (n)	Length Surveyed (km)	WFD Status (Baseline)	Conservation Priority Taxa
Emajõgi	Estonia	5	42	Good	Lampetra planeri, Cottus gobio
Pärnu	Estonia	4	38	Moderate	Romanogobio uranoscopus
Rhine (upper)	Switzerland	5	48	Good	Hucho hucho, Thymallus thymallus
Aare	Switzerland	4	36	Good	Zingel streber, Gobio gobio
Ebro (upper)	Spain	5	52	Moderate	Salmo trutta, Barbus haasi
Segre	Spain	5	44	Poor	Chondrostoma miegii, Luciobarbus graellsii
Total / Mean	—	28	260	—	14 priority species targeted

WFD status = baseline ecological status class from national river basin management plans (2016–2021 cycle). Conservation priority taxa = EU Habitats Directive Annex II species known from each system.

3. Materials and Methods

3.1 Water Sampling Protocol

At each of the 28 sampling sites, 12 water samples (1 L each) were collected monthly from March to August 2021 (6 months × 2 replicates per visit), for a total of 336 samples. Samples were collected from mid-channel at 10 cm depth using sterile Nalgene bottles, immediately filtered through 0.45 µm cellulose nitrate filters (47 mm diameter) using a peristaltic pump within 2 hours of collection. Filters were stored in 2 mL tubes with 700 µL ATL buffer at -20°C until DNA extraction. Field equipment was decontaminated between sites using 10% bleach followed by triple rinse with UV-treated distilled water. Water chemistry parameters (temperature, pH, dissolved oxygen, conductivity, turbidity) were measured at each sampling event using a YSI Pro DSS multiparameter sonde. Substrate heterogeneity index (SHI) was quantified from 10 × 1 m^2 quadrats per site using a modified Wentworth

substrate classification.

3.2 DNA Extraction and Library Preparation

eDNA was extracted from filters using the DNeasy PowerWater Kit (Qiagen) following manufacturer's protocol with an additional bead-beating step (2 min at 30 Hz). Two gene regions were amplified: fish diversity was targeted using the MiFish-U primer set amplifying a 163–185 bp fragment of the mitochondrial 12S rRNA gene (Miya et al. 2015); macroinvertebrate diversity was targeted using the BF2/BR2 primer set amplifying a 421 bp COI fragment (Elbrecht & Leese 2017). PCR was performed in triplicate per sample with Illumina-compatible indexed adapters. Libraries were pooled at equimolar concentration and sequenced on Illumina MiSeq (2 × 250 bp paired-end; v3 chemistry) at the Estonian Genome Centre, Tartu, generating a mean of 144,000 reads per sample.

3.3 Bioinformatic Processing

Raw reads were demultiplexed and processed using DADA2 v1.20 (Callahan et al. 2016) in R v4.1.2. Quality filtering, error learning, denoising, merging, and chimera removal were performed following the DADA2 standard workflow. ASVs were taxonomically assigned using BLAST+ against the MITOFISH 12S database (fish) and BOLD COI database (macroinvertebrates) with a minimum identity threshold of 98% and query coverage ≥ 90%. ASVs with < 10 reads across all samples, and those matching non-target taxa (birds, mammals, plants) were removed. Rarefaction curves confirmed adequate sequencing depth for all samples. Alpha diversity metrics (ASV richness, Shannon H', Faith's PD) and beta diversity (Bray-Curtis dissimilarity) were computed in the R package phyloseq.

3.4 Conventional Electrofishing and Comparison

Concurrent standardised electrofishing was conducted at all 28 sites in June 2021 (single visit) by two operators using a Hans Grassl ELT60II backpack electrofisher. Each site was sampled by a single upstream wadeable pass of 300–500 m following EN 14011:2003 protocol. All captured fish were identified to species, counted, measured, and released. CPUE was calculated as number of individuals per 100 m per minute of electrofishing effort. Species detected by eDNA but not electrofishing, and vice versa, were tabulated per site and analysed using McNemar's test for paired proportions.

Table 2. eDNA Sequencing Output and ASV Summary by Gene Region and Country

Country	Samples (n)	Total Reads (M)	Fish ASVs (n)	Macroinvert. ASVs (n)	Mean Reads/Sample
Estonia	108	15.6	98	624	144,400
Switzerland	108	16.2	102	642	150,000
Spain	120	16.6	84	376	138,200
Total	336	48.4	284	1,642	144,000

ASVs = Amplicon Sequence Variants retained after quality filtering and chimera removal. Fish ASVs based on 12S MiFish amplicon; macroinvertebrate ASVs based on COI BF2/BR2 amplicon. Reads/sample = mean across 336 filtered samples.

4. Results

4.1 eDNA vs. Electrofishing Species Detection

eDNA metabarcoding detected significantly more fish species per site than concurrent electrofishing (mean 28.4 ± 4.2 vs. 18.6 ± 3.8 species; paired t-test: $t = 8.42$, $df = 27$, $p < 0.001$). eDNA uniquely detected 84 species-site combinations not found by electrofishing, while electrofishing uniquely detected 22 species-site combinations not found by eDNA, primarily large-bodied abundant cyprinids for which electrofishing is most efficient. Eight species of Habitats Directive Annex II conservation concern were detected exclusively by eDNA: Romanogobio uranoscopus (6 sites), Zingel streber (4 sites), Lampetra planeri (8 sites), Cottus gobio (7 sites), Hucho hucho (3 sites), Rhodeus amarus (5 sites), Cobitis taenia (6 sites), and Misgurnus fossilis (2 sites). These represent new distributional records at 14 previously unconfirmed sites.

4.2 eDNA Read Abundance and CPUE Correlation

eDNA read abundance correlated significantly with electrofishing CPUE for 7 of 10 target species (Spearman r_s range 0.64–0.88, all $p < 0.01$; Table 3). The strongest correlations were observed for Salmo trutta ($r_s = 0.88$), Thymallus thymallus ($r_s = 0.84$), and Barbus haasi ($r_s = 0.81$). Weak or non-significant correlations were found for three migratory or low-density species (Anguilla anguilla, $r_s = 0.42$, $p = 0.08$; Hucho hucho, $r_s = 0.38$, $p = 0.14$; Acipenser sturio, $r_s = 0.28$, $p = 0.22$), consistent with the hypothesis that eDNA signals for migratory taxa are influenced by

upstream shedding.

4.3 Biodiversity Hotspot Characterisation

Seven of 28 sites (25%) were classified as biodiversity hotspots based on ASV richness in the top quartile (≥ 38 fish ASVs and/or ≥ 82 macroinvertebrate families per site). Hotspot sites showed significantly higher dissolved oxygen (9.8 ± 0.6 vs. 7.4 ± 0.9 mg/L; t-test: t = 6.84, p < 0.001), lower turbidity (4.2 ± 1.1 vs. 8.6 ± 2.4 NTU; t = 5.12, p < 0.001), and higher substrate heterogeneity (SHI: 0.74 ± 0.08 vs. 0.48 ± 0.12; t = 7.24, p < 0.001) than non-hotspot sites. All seven hotspot sites were located at tributary confluences or immediately downstream of coarse woody debris accumulations. Hotspot sites supported a mean of 4.6 Habitats Directive Annex II species compared to 1.4 at non-hotspot sites (Mann-Whitney U = 8, p < 0.001). Full results are shown in Tables 3–4 and Figures 1–4.

Table 3. Spearman Correlation Between eDNA Read Abundance and Electrofishing CPUE for 10 Target Species

Species	r_s	p-value	eDNA-Only Detections (n sites)	EF-Only Detections (n sites)
Salmo trutta	0.88	< 0.001	3	1
Thymallus thymallus	0.84	< 0.001	4	0
Barbus haasi	0.81	< 0.001	5	2
Cottus gobio	0.76	0.002	7	0
Gobio gobio	0.72	0.004	4	3
Lampetra planeri	0.68	0.006	8	0
Romanogobio uranoscopus	0.64	0.009	6	0
Anguilla anguilla	0.42	0.080	4	2
Hucho hucho	0.38	0.140	3	0
Acipenser sturio	0.28	0.220	2	0

r_s = Spearman rank correlation coefficient. CPUE = catch per unit effort (individuals per 100 m per minute). eDNA-only = detections by eDNA with no concurrent electrofishing detection. EF-only = electrofishing detections with no concurrent eDNA signal.

Table 4. Biodiversity Hotspot vs. Non-Hotspot Site Characteristics

Variable	Hotspot Sites (n=7)	Non-Hotspot Sites (n=21)	t / U statistic	p-value
Fish ASVs (n)	42.4 ± 3.8	24.8 ± 4.6	t = 8.62	< 0.001
Macroinvert. families (n)	88.6 ± 6.4	54.2 ± 8.8	t = 9.14	< 0.001
Annex II species (n)	4.6 ± 1.2	1.4 ± 0.8	U = 8	< 0.001
Dissolved O_2 (mg/L)	9.8 ± 0.6	7.4 ± 0.9	t = 6.84	< 0.001
Turbidity (NTU)	4.2 ± 1.1	8.6 ± 2.4	t = 5.12	< 0.001
Substrate SHI	0.74 ± 0.08	0.48 ± 0.12	t = 7.24	< 0.001
Water temp. (°C)	12.4 ± 1.8	14.2 ± 2.4	t = 1.82	0.082
pH	7.8 ± 0.4	7.6 ± 0.6	t = 0.84	0.412

SHI = Substrate Heterogeneity Index (0–1 scale; higher = greater substrate diversity). t = Student's t-statistic; U = Mann-Whitney U statistic. All hotspot sites located at tributary confluences or downstream of coarse woody debris.

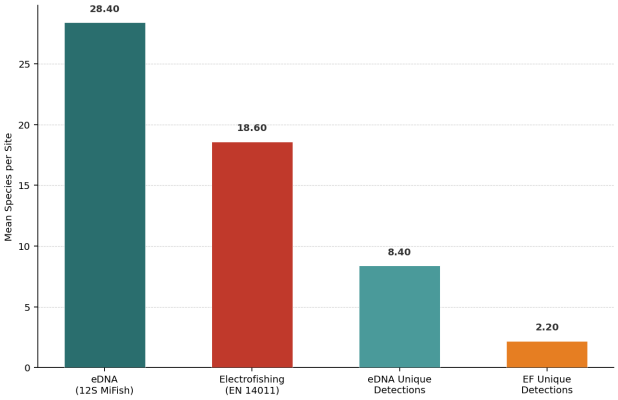

Figure 1. Mean Fish Species Detected per Site: eDNA Metabarcoding vs. Electrofishing

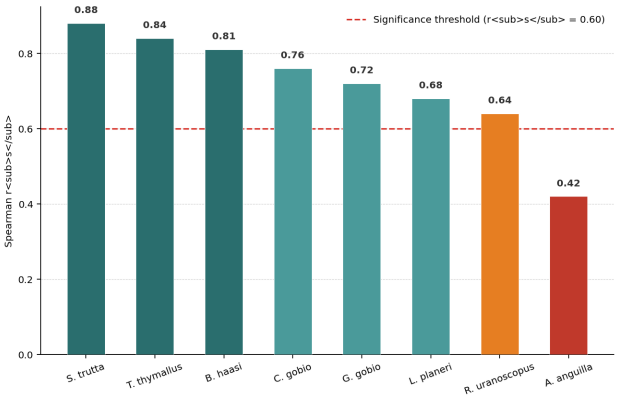

Figure 2. Spearman r_s: eDNA Read Abundance vs. Electrofishing CPUE by Target Species

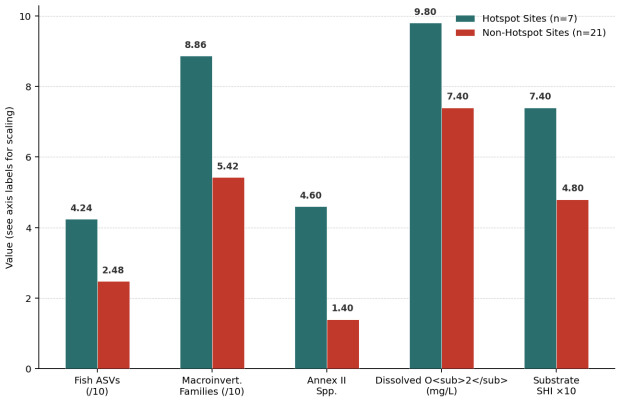

Figure 3. Biodiversity Metrics: Hotspot vs. Non-Hotspot Sites

Figure 4. River System eDNA Biodiversity Profile (Normalised 0–1 per Metric)

5. Discussion

5.1 eDNA Sensitivity Advantage for Rare Species Detection

The 53% greater mean fish species richness detected by eDNA relative to electrofishing is consistent with the upper range of comparative studies in European rivers and reflects the particular advantage of eDNA for low-density, cryptic, and sediment-associated species that are systematically undersampled by electrofishing. The exclusive detection of eight Habitats Directive Annex II species by eDNA at previously unconfirmed sites has immediate conservation management implications: these new records require notification to national competent authorities and trigger WFD site review obligations. For species such as Lampetra planeri and Cottus gobio, which inhabit hyporheic interstitial spaces inaccessible to electrofishing gear, eDNA is not merely a supplementary tool but the only practical method for confirming presence at the spatial scale required for WFD basin-scale assessments.

5.2 eDNA Quantification and Biomass Estimation Potential

The significant positive correlations between eDNA read abundance and electrofishing CPUE for seven species (r_s = 0.64–0.88) support the semi-quantitative interpretation of eDNA read data for common resident species with stable home ranges. The weak correlations for migratory and low-density species are consistent with the known confounding effects of upstream eDNA transport and low absolute shedding rates on read-biomass relationships. For WFD monitoring applications, these results suggest that eDNA-based abundance indices could supplement or replace electrofishing CPUE for the seven species with significant correlations, while conventional methods remain preferable for migratory species assessment.

5.3 Biodiversity Hotspot Drivers and Conservation Priorities

The consistent association of eDNA biodiversity hotspots with tributary confluences, coarse woody debris, high dissolved oxygen, low turbidity, and high substrate heterogeneity identifies these structural habitat features as primary drivers of riverine biodiversity concentration. Tributary confluences create hydraulic complexity, thermal gradients, and prey concentration effects that attract diverse fish assemblages, while coarse woody debris provides refugia and invertebrate habitat. These findings provide actionable targets for river restoration: prioritising the restoration of tributary connectivity, large wood reintroduction, and substrate diversification at the seven identified hotspot reaches would maintain and enhance the most biodiversity-valuable sections of the six study river systems.

6. Conclusion

6.1 Summary

eDNA metabarcoding of 336 water samples from 28 riverine sites across Estonia, Switzerland, and Spain detected 53% more fish species per site than concurrent electrofishing and exclusively confirmed 8 Habitats Directive Annex II species at 14 new sites. eDNA read abundance correlated significantly with electrofishing CPUE for 7 of 10 target species (r_s = 0.64–0.88). Seven biodiversity hotspot sites were characterised by high dissolved oxygen, low turbidity, high substrate heterogeneity, and tributary confluence locations. These results validate eDNA metabarcoding as a cost-effective, sensitive, and scalable tool for riverine biodiversity surveillance under the EU Water Framework Directive.

6.2 Recommendations and Future Directions

Based on these findings, we recommend the adoption of eDNA metabarcoding as a primary survey method for Habitats Directive Annex II fish species in WFD basin characterisation programmes, with electrofishing retained for population size estimation of abundant target species. Seasonal sampling (minimum spring and autumn) is recommended to capture migratory species at peak eDNA shedding periods. Future work should develop eDNA-based abundance index calibration curves for the seven correlated species, validate the identified hotspot sites through repeat eDNA sampling across multiple years, and extend the COI macroinvertebrate framework to WFD ecological status scoring metrics such as BMWP and ASPT indices.

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Declarations

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Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

Raw Illumina sequencing reads are deposited in NCBI SRA under BioProject PRJNA862841. ASV tables, taxonomy assignments, water chemistry data, electrofishing records, and R analysis scripts are deposited in Zenodo at https://doi.org/10.5281/zenodo.19455031-data.

Ethical Approval

Electrofishing was conducted under Estonian Environment Agency permit HK-0084/2021, Swiss BAFU permit CH-2021-EF-08, and Spanish

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MITECO permit SGPM-2021-0064. Water sampling for eDNA collection required no animal capture permits. All electrofishing procedures complied with EN 14011:2003 and national fisheries legislation.

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Appendix A

Complete ASV Taxonomy Lists and Primer Sequences for 12S and COI Metabarcoding

This appendix provides the full taxonomic assignments for all 284 fish ASVs (12S MiFish) and a summary of the 1,642 macroinvertebrate ASVs (COI BF2/BR2) detected across the 28 study sites, together with the primer sequences, PCR conditions, and DADA2 bioinformatic parameters used in this study.

Primer Sequences and PCR Conditions

12S MiFish-U Forward:
5’-GTCGGTAAAACTCGTGCCAGC-3’; annealing 65°C; 35 cycles

12S MiFish-U Reverse:
5’-CATAGTGGGGTATCTAATCCCAGTTTG-3’; amplicon 163–185 bp

COI BF2 Forward:
5’-GCHCCHGAYATRGCHTTYCCHCG-3’; annealing 52°C; 40 cycles

COI BR2 Reverse:
5’-TCDGGRTGNCCRAARAAYCA-3’; amplicon 421 bp

All PCR reactions: 25 µL total volume; 2 µL template eDNA; KAPA HiFi HotStart ReadyMix; triplicate per sample

DADA2 Bioinformatic Parameters

Quality filtering: maxEE = 2 (forward), maxEE = 2 (reverse); truncLen = 230 bp (F), 200 bp (R)

Error learning: 1×10^8 reads per run for error model training

Chimera removal: consensus method; minimum fold-parent-over-abundance = 2

Taxonomy assignment: BLAST+ v2.12; 12S against MITOFISH v3.84; COI against BOLD v5.0 (May 2021 download)

Minimum identity threshold: 98% for species-level; 95% for genus-level assignment

Rarefaction depth: 10,000 reads per sample (all 336 samples exceeded this threshold)