

Article

Assessment of Freshwater Unionidae Using Environmental DNA Metabarcoding in Lentic Ecosystems: Implications for Spatial Sampling Strategies

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Simple Summary

Freshwater mussels play an important role in maintaining the health of lake ecosystems, but they are difficult to monitor because they live buried in sediments and are hard to detect using traditional survey methods. This study evaluated the effectiveness of eDNA metabarcoding in detecting freshwater Unionidae mussels in lake water columns and examined their spatial and seasonal distribution patterns. Water samples were collected from the edges and the central areas of four lakes during autumn and winter, from the surface, middle, and bottom layers. The results showed that mussel genetic signals were consistently detected in all lakes, even without direct observation of the animals. Overall detection did not change greatly between seasons, but clear differences were found among sampling locations. Genetic signals were lower near the shoreline and higher in central lake areas, while water depth within the central zone had little effect. Two common mussel species showed different spatial patterns, reflecting their habitat preferences rather than seasonal activity. These findings demonstrate that water-based genetic monitoring can provide an efficient, non-invasive means to assess freshwater mussel diversity in lakes. The study offers practical guidance for designing lake monitoring programs that support biodiversity conservation and ecosystem management.

Abstract

Environmental DNA (eDNA) metabarcoding has become a powerful, non-invasive method for detecting aquatic organisms. However, optimal sampling strategies for benthic taxa in lentic ecosystems remain unclear. This study evaluated the effectiveness of eDNA metabarcoding in detecting freshwater Unionidae mussels in lake water columns and examined their spatial and seasonal distribution patterns. We validated a mini-barcode primer targeting the mitochondrial 16S rDNA of unionid mussels through controlled laboratory experiments and field tests, confirming reliable amplification and accurate taxonomic assignment of freshwater bivalve DNA. Field surveys were conducted in four lakes within the Nakdong River basin, where eDNA samples were collected from littoral zones and from surface, mid-, and bottom layers of central lake areas during autumn and winter. Metabarcoding analysis identified 79 amplicon sequence variants (ASVs) representing four unionid taxa, with *Cristaria plicata* and *Sinanodonta lauta* comprising the majority of reads and ASVs. Overall, the number of Unionidae eDNA reads showed no significant seasonal differences, but there was notable spatial variation among sampling locations. Read numbers were significantly lower in littoral zones compared to central lake

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areas, with no significant differences detected among depth layers within the central zones. Species-specific analyses revealed contrasting spatial patterns: *C. plicata* had higher read abundance in mid- and bottom layers, while *S. lauta* was more frequently detected in surface and littoral samples. These findings suggest that the distribution of freshwater mussel eDNA in lakes is primarily influenced by spatial factors related to habitat preference and hydrodynamic mixing, rather than by seasonal variation during stable periods. This study offers practical insights for designing effective eDNA sampling strategies for benthic invertebrates in lentic ecosystems.

Keywords: environmental DNA (eDNA); freshwater mussels; unionidae; lentic ecosystems; metabarcoding; spatial distribution; sampling strategy

1. Introduction

Environmental DNA (eDNA) is genetic material from organisms that can be found in different environmental contexts, including water, soil, and air. It carries genetic information from a variety of organisms within a specific environment [1–3]. In aquatic systems, eDNA typically exists in an intracellular form, released when somatic cells are shed from organisms. This genetic material can be effectively concentrated using water filtration techniques. Due to this capability, eDNA-based approaches have gained significant attention as a way to address the limitations of traditional organism-based sampling methods in aquatic environments [1,4]. Furthermore, for species that are challenging to capture directly because of low population densities, eDNA offers a highly eco-friendly monitoring tool that allows for the assessment of species presence with minimal disturbance to their habitats [5,6].

Although environmental DNA (eDNA) has been widely used worldwide to explore biodiversity across various taxonomic groups, the significance of eDNA sampling strategies for assessing species diversity has received relatively limited attention compared to other factors. In lotic ecosystems, water flows from upstream to downstream, influencing the horizontal distribution of eDNA within the water body [7,8]. In contrast, lentic ecosystems, such as reservoirs and lakes, are characterized by greater water depth and limited water movement, which allows for the formation of thermal stratification that varies with the seasons [9]. This stratification can influence the vertical distribution of eDNA, suggesting that eDNA sampling in lakes should account for both horizontal and vertical spatial heterogeneity. In particular, benthic invertebrates, such as freshwater bivalves, inhabit the substrate and exhibit slow movement within restricted spatial ranges, potentially leading to highly localized eDNA distributions [10]. These characteristics indicate that eDNA sampling strategies may need to be customized based on the habitat preferences and ecological traits of various taxonomic groups.

In freshwater bivalves found in lentic ecosystems, species in the order Unionoida typically burrow into the muddy substrates of lake bottoms. Due to their extremely low mobility and infrequent presence in littoral zones, studies on the population distribution and diversity of this group face significant challenges [11]. As a result, conventional surveys of unionid mussels have relied on labor-intensive methods, such as hand or rake collection from muddy substrates during diving, along with dredging in nearshore areas and using grab samplers in deeper central zones [12]. In contrast, eDNA-based approaches facilitate the detection of unionid mussels through genetic material present in the water column, potentially offering higher detection probabilities and greater survey efficiency than traditional field methods.

Accordingly, this study developed three hypotheses to assess the applicability and limitations of using environmental DNA (eDNA) to detect freshwater bivalves in lentic ecosystems. First, mini-barcode primers tailored for freshwater bivalves can effectively detect and identify genetic material from this taxonomic group in eDNA found in the water column. Second, the distribution patterns of freshwater bivalve eDNA show spatial variability based on sampling locations within a lake, including the littoral zones and the different layers of the central area (surface, middle, and bottom). Third, the distribution characteristics of freshwater bivalve eDNA vary seasonally in response to changes in hydrological and physical environmental conditions. This study aims to compare and analyze the spatiotemporal distribution patterns of freshwater bivalve eDNA in lake water bodies, providing essential insights for developing effective eDNA sampling strategies for bivalves in lentic environments.

2. Materials and Methods

2.1. Primer Validation for Bivalve eDNA Detection

To determine if the mini-barcode primers used in this study could effectively amplify genetic material from freshwater bivalves in water, a primer validation experiment was conducted under controlled laboratory conditions with three replicates. Individual *Sin-nodonta woodiana* of similar sizes were placed in plastic tanks containing 3 L of sterile primary distilled water, which were then covered with plastic lids to prevent contamination. The tanks were continuously aerated for 24 h to promote the release of somatic cells from the bivalves into the water. After 24 h, the *A. woodiana* individuals were removed, and the remaining tertiary distilled water in each tank was collected for eDNA analysis.

2.2. Field Sampling

To determine if the mini-barcode primers could successfully identify freshwater bivalve species using eDNA from natural water bodies, we collected water-column eDNA samples from two shallow sites (GUn and CJs) where bivalve individuals were directly observable (Figure 1). These sites were chosen exclusively for field validation of the primers and were excluded from any comparative analyses between lakes or water layers.

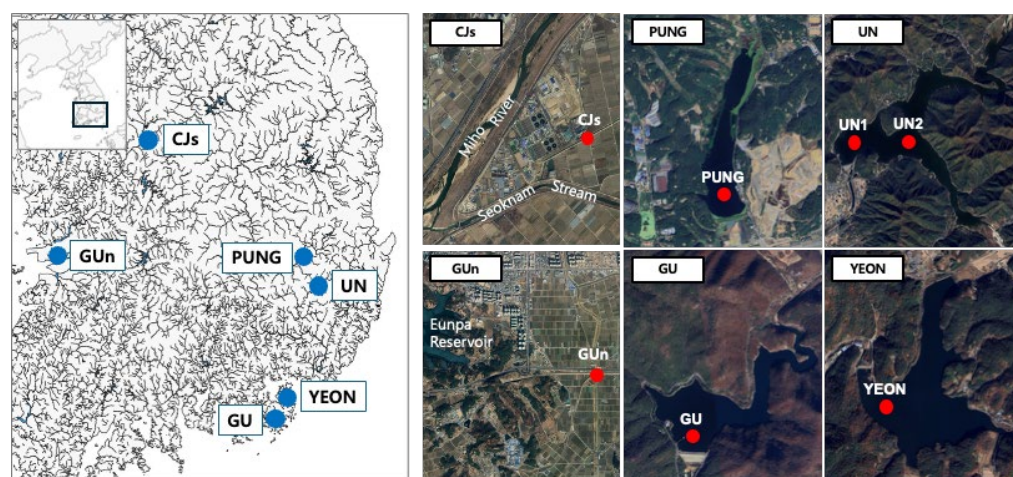


Figure 1. Geographic overview of sampling sites for Unionidae eDNA analysis. The map of South Korea (**left**) highlights the locations of six study sites (blue dots). CJs and GUn are stream sites (lotic environments), while the remaining four sites are lakes or reservoirs (lentic environments). Enlarged satellite images (**right**) indicate the surveyed lakes (red dots). According to NAEMP guidelines, two sites (littoral and deep-water zones) were sampled in the medium-sized lake (UN), whereas one representative site was sampled in each of the small lakes (PUNG, GU, and YEON). To compare the spatial and seasonal distribution patterns of freshwater bivalve eDNA, we

conducted repeated eDNA sampling in four lakes (PUNG, UN, GU, and YEON) within the Nakdong River basin watershed. Sampling took place once in autumn (October 2024) and once in winter (February 2025) during periods when water-level fluctuations were minimal. Water samples were collected separately from the littoral and central zones, with samples from the central zone further divided into surface, middle, and bottom layers. During each sampling campaign, 1 L of water was collected from each designated depth, ensuring consistency across surveys.

Vertical water layers were defined based on relative depth profiles following the National Aquatic Ecological Monitoring Program (NAEMP) guidelines. Surface (upper) samples were collected at approximately 0.5 m below the water surface, middle-layer samples at mid-depth of the water column, and bottom (lower) samples at approximately 0.5–1.0 m above the sediment–water interface. This stratification was adopted to capture potential vertical heterogeneity in eDNA distribution while minimizing the influence of sediment resuspension. Detailed lake depth information and corresponding sampling depths for each site are provided in Table S1.

Lakes were compared using independent replicates, while comparisons among water layers and sampling locations within the same lake were analyzed through a repeated-measures framework. This approach enabled an evaluation of the relative impacts of water depth and seasonal factors on freshwater bivalve eDNA distribution, accounting for environmental differences among lakes.

To reduce the risk of cross-contamination during sampling, the interior of the water sampler was rinsed with 20% diluted sodium hypochlorite before each collection, followed by a thorough rinse with site water to eliminate any residual disinfectant. In accordance with the National Aquatic Ecological Monitoring Program (NAEMP) guidelines from the Ministry of Environment, water samples were collected from two sites in the medium-sized lake (UN) and from one site in each of the small lakes (PUNG, GU, and YEON) [13] (Table S1). A Van Dorn water sampler (Wildco®, Yulee, FL, USA) was used to gather water from each depth, and the samples were then transferred to sterile sampling bottles (Newkukje Scientific Corporation, Namyangju, Republic of Korea) for transport to the laboratory under cold and dark conditions.

2.3. eDNA Filtration, Concentration, and Extraction

Water samples were collected and filtered using a vacuum pump (DOA-P704-AC, Gast Manufacturing, Inc., Benton Harbor, MI, USA) along with glass fiber filters (GF/F: pore size 0.7 µm, diameter 47 mm; Whatman, Maidstone, UK), which have been widely used for aquatic eDNA collection in previous studies [14,15]. For the laboratory validation experiment, 100 mL of water was filtered; for field-collected samples, 500 mL was filtered. A filtration volume of 500 mL was selected to balance filtration efficiency and clogging risk under turbid field conditions. To monitor potential contamination during filtration, a filtration blank was included by filtering nuclease-free distilled water instead of environmental water using the same filtration apparatus and procedures as for field samples.

After filtration, each filter containing concentrated eDNA was placed in a silica bead tube (Qiagen Co., Hilden, North Rhine-Westphalia, Germany) and stored at −80 °C until extraction. eDNA extraction from the stored filters was carried out following the manufacturer's protocol with the DNeasy® PowerWater® Kit (Qiagen Co., Hilden, Germany). The extracted eDNA was then stored at −80 °C until further analysis. To minimize the risk of contamination, all procedures were performed with sterilized equipment and consumables. Filtration devices and tools were thoroughly cleaned before use, and all steps were conducted in a dedicated laboratory space, physically separated from post-PCR areas. These precautions were implemented to reduce the risk of external DNA contamination during sample processing.

2.4. eDNA Amplification and Sequence Analysis

To enhance the bivalve-derived genetic material in eDNA samples, we utilized a mini-barcode primer set (Unio16S-F/R) designed to target the mitochondrial 16S rRNA gene (mitochondrial 16S rDNA) of freshwater bivalves [16]. The first-round PCR reaction mixture included 10 µL of DNA polymerase premix (2× GainBlue™ Hot Start Max Master Mix, GainBio, Daejeon, Republic of Korea), 1 µL each of forward and reverse primers at 10 pmol/µL, and 5 µL of molecular-grade sterile tertiary distilled water. Additionally, 3 µL of template eDNA was added to each reaction.

PCR amplification commenced with an initial denaturation step at 95 °C for 3 min, followed by 40 cycles consisting of denaturation at 95 °C for 1 min, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. A final extension step was performed at 72 °C for 3 min. Each sample was amplified in two to three technical replicates. Negative controls containing nuclease-free water instead of template DNA were included in each PCR run for both indoor reference barcode amplification and field-derived eDNA amplification. No Unionidae reads were detected in any PCR-negative controls.

PCR products were visualized using an E-Gel™ Power Snap Electrophoresis System (Thermo Fisher Scientific, Waltham, MA, USA) on 2% agarose gels. Images were captured with an E-Gel™ Power Snap Camera (Thermo Fisher Scientific, Waltham, MA, USA) to confirm the presence of target amplicons (180–190 bp) and to identify any non-specific amplifications. To eliminate non-specific products before downstream sequencing and eDNA metabarcoding analyses, target-sized amplicons were excised using a Size Selection Gel (Thermo Fisher Scientific, Waltham, MA, USA).

Purified amplicons were sequenced by a commercial service provider (BioFact Co., Daejeon, Republic of Korea) using the Sanger sequencing method. The resulting sequences were analyzed alongside reference sequences of freshwater bivalve species found in Korea using the maximum likelihood (ML) method implemented in MEGA 12 (version 12.1.1) software. Bootstrap support values (≥50%) were calculated based on 1000 replicates, and the analysis was conducted under the Tamura-Nei nucleotide substitution model [17].

2.5. Library Preparation and Metabarcoding Analysis

For library preparation, second-round PCR was conducted using pooled first-round PCR products from two technical replicates. Illumina MiSeq libraries were created by attaching dual indices with the Nextera XT Index Kit v2. The second-round PCR involved an initial denaturation at 95 °C for 3 min, followed by 8 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, concluding with a final extension at 72 °C for 5 min.

The prepared freshwater mussel eDNA metabarcoding libraries were sequenced by MacroGen Co., a commercial provider in Sejong City, Republic of Korea. This study focused on a targeted eDNA approach for metabarcoding analyses, specifically examining freshwater unionid mussels. The analytical workflow was designed to assess the detection and relative read distribution of Unionidae taxa, rather than to characterize broader community-level diversity.

Raw paired-end reads generated using the Illumina platform were processed with Cutadapt (v3.2) to remove adapter and primer sequences. The forward and reverse reads were trimmed to 190 bp and 150 bp, respectively. Error correction, read merging, and denoising were subsequently carried out using DADA2 (v1.18.0) [18], excluding reads with expected error values greater than 2. Chimeric sequences were identified and removed using the consensus method implemented in the removeBimeraDenovo function of DADA2. Amplicon sequence variants (ASVs) were taxonomically assigned using BLAST+ (v2.9.0) against the NCBI nucleotide (NT) database. Due to the primer specificity and the relatively short length of the amplified region, only alignments with query

coverage $\geq 85\%$ and sequence identity $\geq 85\%$ were retained for downstream analyses. These criteria supported taxonomic assignment within Unionidae but did not establish definitive species-level identifications. To address differences in sequencing depth among samples, normalization was performed using QIIME (v1.9) by rarefying all samples to the minimum read count. This procedure minimized potential bias associated with uneven sequencing depth when comparing the relative distribution of unionid reads across samples. The resulting ASV table was used for subsequent statistical analyses and data visualization.

Metabarcoding results were analyzed by examining ASVs and read sequences based on sampling period and location using Microsoft Excel (Microsoft, Redmond, WA, USA). Data visualization was conducted in the R environment (version 4.4.3; R Core Team, 2025) using RStudio (version 2025.05.0; Posit Team, Boston, MA, USA), with graphical outputs generated using the ggplot2 package [19].

2.6. Statistical Analysis

To compare the spatial and seasonal distribution patterns of freshwater bivalve eDNA, this study treated each lake as an independent, replicated ecosystem. Samples were collected from four lakes (PUNG, UN, GU, and YEON) within the same watershed, focusing on the littoral zones and the surface, mid-, and bottom layers of the central zones during autumn and winter.

Statistical analyses were performed on individual samples categorized by sampling season, location, and water layer. Since repeated samples collected within the same lake shared similar environmental conditions, the analysis accounted for this within-lake repeated-measures structure during interpretation.

The explanatory factors included sampling season (autumn, winter), sampling location (littoral zone, central zone), and water layer (surface, middle, bottom) in the central zone. The response variables were the total read counts of Unionidae, read counts of dominant species (*Cristaria plicata* and *Sinanodonta lauta*), and the number of Unionidae amplicon sequence variants (ASVs). Due to the limited number of replicated ecosystems and violations of normality and homoscedasticity assumptions, non-parametric statistical methods were applied for group comparisons. Differences in distributions among groups were assessed using the Kruskal–Wallis test, with a significance threshold set at $p < 0.05$ [20]. To enhance p -value-based inference and evaluate both the magnitude and direction of group differences, Cliff's delta (δ) was calculated as a measure of effect size, with the following classifications: $|\delta| < 0.147$ indicates a small effect, $0.147 \sim 0.33$ indicates a medium effect, and ≥ 0.33 indicates a large effect [21].

In addition, one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test was applied for exploratory pairwise comparisons among sampling locations and water layers to facilitate group-wise comparison of mean differences.

All statistical analyses were conducted in the R environment (version 4.4.3) using R Studio, and data visualization was carried out using the ggplot2 package.

3. Results

3.1. Validation of Mini-Barcode Primers for Bivalve Gene Amplification

Analysis of PCR amplicons from *Sinanodonta woodiana* generated in laboratory tank experiments revealed that target-sized PCR products (180–190 bp) were present in all samples. Consistent, strong bands of the same size were observed across all three replicates (Figure 2), with no differences in band migration among the samples. While some samples

exhibited weak non-specific amplification products, these were clearly distinguishable from the target-sized amplicons.

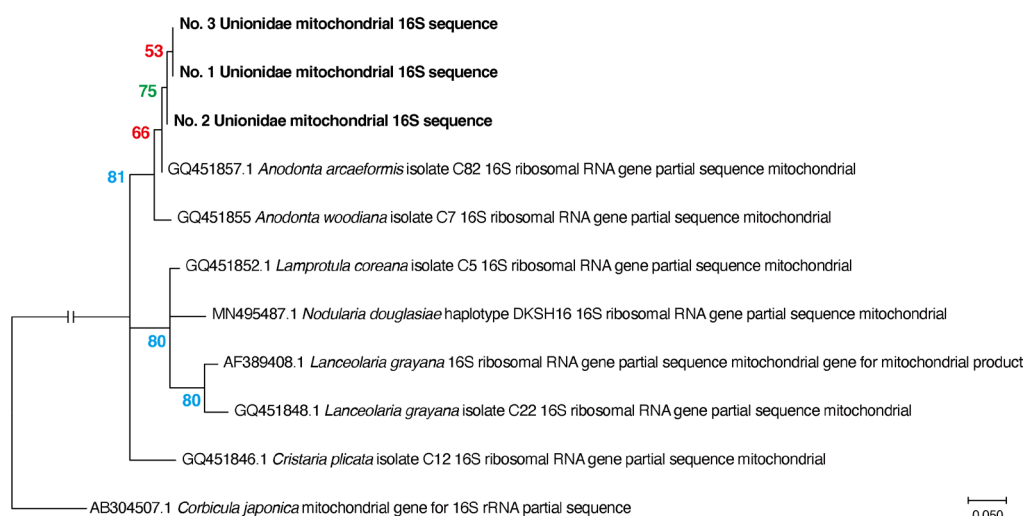


Figure 2. Phylogenetic relationships among Unionidae species were inferred from mitochondrial 16S rRNA gene sequences. Bootstrap support values ($\geq 50\%$) are indicated at the nodes. Values of 100–80% were indicated in blue, 79–70% in green, and values below 70% in red. The scale bar represents the number of nucleotide substitutions per site. Unionidae sequences generated in this study are highlighted, and GenBank accession numbers are provided for reference sequences.

Phylogenetic analysis of the amplified sequences revealed that the amplicons clustered into a single clade with *A. woodiana* (*Sinanodonta woodiana*) sequences previously reported from Korea, distinctly separating them from other freshwater bivalve species found in Korea, such as *Nodularia douglasiae*, *Lamprotula coreana*, *Lanceolaria grayana*, and *Cristaria plicata*.

Validation of the primers using field-collected samples successfully amplified the mitochondrial 16S rDNA of freshwater bivalves, corroborating the results from laboratory experiments (Figure 3). A single amplicon of the expected target size (approximately 210 bp, which includes a primer amplicon of 180–190 bp and an index consensus sequence of about 20 bp) was detected (Figure S1). In the GUn samples, 67,137 reads were obtained, with *Sinanodonta lauta* representing the highest proportion at 74%. Additionally, sequences corresponding to *Nodularia douglasiae*, *Sinanodonta schrenkii*, *Lanceolaria grayii*, and *Cristaria plicata* were also identified. In the CJs samples, a total of 67,277 reads were obtained, similar to those from GUn; however, only sequences classified as *S. lauta* (97%) and *N. douglasiae* (3%) were detected, with no sequences corresponding to *C. plicata*.

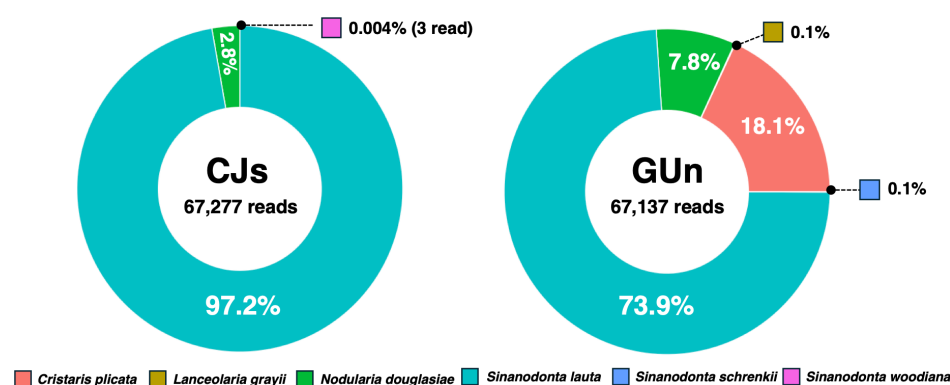


Figure 3. Relative read abundance of Unionidae species detected by eDNA metabarcoding at two stream sites, CJs and GUn, is presented. Donut charts illustrate the proportion of mitochondrial 16S

rRNA gene reads assigned to each Unionidae species at each site, with the total number of reads indicated at the center of each chart. Species are color-coded according to the legend.

3.2. eDNA Amplification and Unionidae ASV Analysis

Metabarcoding analysis of samples collected from the four lakes resulted in a total of 108,997 reads, identifying 79 amplicon sequence variants (ASVs). These ASVs were assigned to four freshwater bivalve taxa: *Cristaria plicata*, *Sinanodonta lauta*, *Cristaria truncata*, and *Sinanodonta* cf. (Table S2). Among these, *C. plicata* comprised the largest proportion, accounting for 54.4% of the total ASVs (43 ASVs), followed by *S. lauta* with 35.4% (28 ASVs). In contrast, *C. truncata* and *Sinanodonta* cf. had relatively few ASVs, with 5 ASVs (6.3%) and 3 ASVs (3.8%), respectively. Read-based analysis showed a similar pattern, with *C. plicata* and *S. lauta* together representing over 90% of the total Unionidae reads.

During the autumn and winter sampling periods, 49 and 45 ASVs were detected, respectively, indicating no significant seasonal difference in overall ASV richness (Figure 4A). The species-level ASV composition mirrored this pattern, with *C. plicata* contributing the highest number of ASVs in both seasons, followed by *S. lauta*. Conversely, *C. truncata* and *Sinanodonta* cf. were represented by fewer than five ASVs in both autumn and winter.

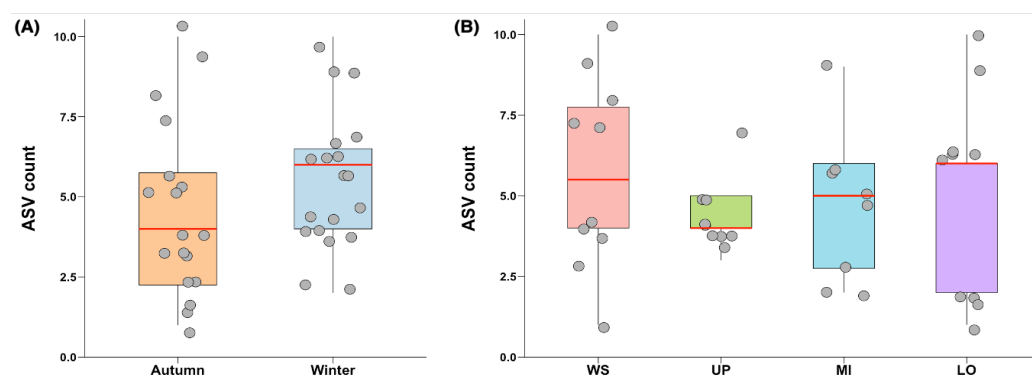


Figure 4. Boxplots illustrating the variation in amplicon sequence variant (ASV) richness of Unionidae across (A) seasons and (B) sampling positions (WS: littoral zone, UP: surface layer, MI: middle layer, LO: bottom layer). Box colors represent the season and sampling position shown on the x-axis. The boxes represent the interquartile range (IQR), the horizontal red line indicates the median, and the whiskers extend to 1.5× IQR. Individual points represent the observed ASV counts for each sample.

Differences in ASV richness were observed among sampling locations (Figure 4B). The highest number of ASVs was found in littoral samples, with 39 ASVs, followed by bottom-layer samples with 33 ASVs. In contrast, surface samples had 26 ASVs, and mid-water layers had 21 ASVs. While the number of ASVs for individual species differed by location, *C. plicata* consistently represented the largest proportion of ASVs across all areas. In the littoral zone, *C. plicata* had the highest count, differing from *S. lauta* by just one ASV. In the central zone of the lakes (surface, mid, and bottom layers), the difference in ASV numbers between *C. plicata* and *S. lauta* was smallest in the mid-water layer (3 ASVs) and largest in the bottom layer (8 ASVs).

3.3. Differences in Unionidae Read Numbers by Season and Sampling Location

Across sampling seasons, the overall distribution range of Unionidae read numbers was broader in autumn than in winter (Figure 5A), although no statistically significant difference was found between the two seasons (Kruskal–Wallis test, $p > 0.05$). In autumn, read numbers varied widely, with a minimum of 3 reads and a maximum of 4300 reads, yielding a median of 2686 reads and a mean of 2469 reads. Both high counts exceeding

4000 reads and low counts below 2000 reads were recorded during this season. In winter, read numbers also displayed a wide range, from 10 to 4300 reads, similar to autumn. However, the majority of reads fell between 2000 and 4300, resulting in a higher median (3143 reads) and mean (2932 reads) compared to autumn.

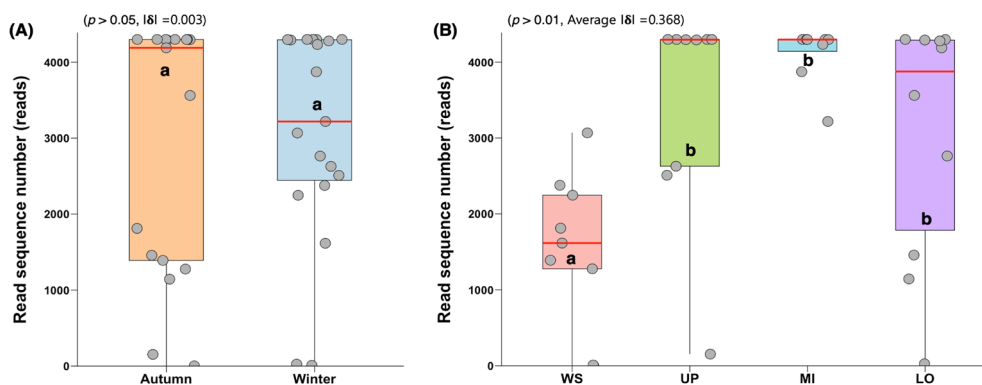


Figure 5. Boxplots illustrate the variation in Unionidae read abundance across (A) seasons (Autumn and Winter) and (B) sampling positions (WS: littoral zone, UP: surface layer, MI: middle layer, LO: bottom layer). Box colors represent the season and sampling position shown on the x-axis. The boxes represent the interquartile range (IQR), red horizontal lines indicate median values, and whiskers denote 1.5× IQR. Gray dots correspond to individual sample values. Different lowercase letters above the boxes indicate statistically significant pairwise differences among groups, as determined by one-way ANOVA followed by Tukey’s HSD post hoc test ($p < 0.05$).

Evaluation of read number differences among sampling locations revealed that littoral zone (WS) samples had lower read counts than samples from the central zone of the lakes (Figure 5B). The littoral zone (WS) samples had a mean of 1380 reads and a median of 1616 reads, while samples from the central zone exhibited a mean of 3141 reads and a median of 4156 reads. Box plot comparisons indicated that both the median and interquartile range (IQR) of littoral zone (WS) samples were consistently lower than those of all depth layers (upper, middle, and lower) in the central zone.

Statistically significant differences were found between littoral zone (WS) samples and both the upper and middle layers of the central zone (littoral zone-surface: $p = 0.006$; littoral zone-middle: $p = 0.001$). A significant difference was also identified between littoral zone (WS) and lower-layer samples, though this difference was smaller than those observed for the upper and middle layers ($p = 0.045$).

Cliff’s delta (δ) analysis revealed a medium effect size between littoral zone and surface-layer samples ($\delta = 0.36$), while large effect sizes were noted between the littoral zone and both the middle ($\delta = 0.81$) and bottom layers ($\delta = 0.82$).

In contrast, no statistically significant differences in read numbers were found among the depth layers within the central zone of the lakes. This finding was supported by Kruskal–Wallis test results (surface-middle: $p = 0.727$; surface-bottom: $p = 0.263$; middle-bottom: $p = 0.116$) and Cliff’s delta analyses, which indicated no large effect sizes among the depth layers. The surface and bottom layers showed broad distributions, with mean read counts of 3108 and 3031, respectively. The middle layer had a similar mean (3282 reads) and median (4299 reads), but exhibited a relatively narrower distribution.

3.4. Differences in Read Numbers of Dominant Species by Sampling Location

Sequences for *Cristaria plicata* and *Sinanodonta lauta* were found at all sampling sites, with these two species together comprising over 80% of the total Unionidae reads. The overall abundance of reads was higher for *C. plicata* (mean of 1867 reads) compared to *S.*

lauta (mean of 834 reads). Boxplot visualizations showed that the read number distributions for both species largely overlapped between autumn and winter (Figure 6A). Seasonal differences in read distributions for *C. plicata* were minimal, while *S. lauta* exhibited more variability between seasons. However, the seasonal differences in read numbers were not statistically significant for either species (Kruskal–Wallis test, $p > 0.05$). Cliff's delta (δ) analysis indicated a small effect size for the seasonal differences in *S. lauta* read numbers ($|\delta| = 0.298$).

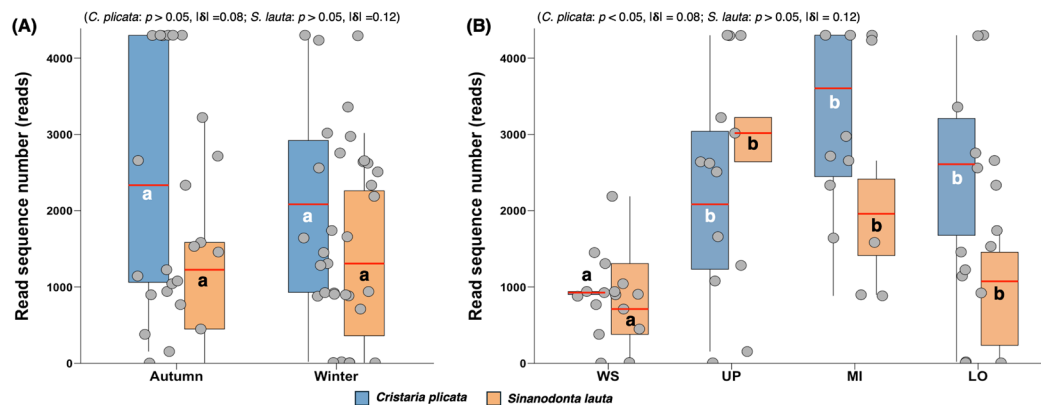


Figure 6. Boxplots show seasonal and spatial variation in read abundance for two Unionidae species, *Cristaria plicata* and *Sinanodonta lauta*. Panel (A) compares read numbers between Autumn and Winter, while panel (B) compares read numbers across sampling positions (WS: littoral zone, UP: surface layer, MI: middle layer, LO: bottom layer). Boxes represent the interquartile range (IQR), red horizontal lines indicate median values, and whiskers denote $1.5 \times$ IQR. Gray dots correspond to individual samples. Different lowercase letters within each species indicate statistically significant pairwise differences among groups, as determined by one-way ANOVA followed by Tukey's HSD post hoc test ($p < 0.05$).

With respect to sampling location, 67.2% of the total reads of *C. plicata* were detected in the middle layer (median: 3604 reads) and bottom layer (median: 2609 reads) of the central zone, while 23.9% were detected in the surface layer (median: 2084 reads) (Figure 6B). In contrast, *S. lauta* exhibited a different spatial pattern, with 61.2% of its total reads found in the surface layer of the central zone (median: 3017 reads) and in littoral samples. The remaining reads of *S. lauta* were distributed across the middle and bottom layers, accounting for 22.4% and 16.2% of the total reads, respectively. Thus, the sampling locations associated with higher read numbers differed between species, with *C. plicata* showing greater abundance in the middle and bottom layers, whereas *S. lauta* exhibited the highest abundance in the surface layer. Differences in read numbers among sampling locations were statistically significant only for *C. plicata* ($p < 0.05$). However, Cliff's delta (δ) indicated large effect sizes for spatial differences in both species. For *C. plicata*, the middle-water layer showed the largest differences compared to other locations (surface: $|\delta| = 0.39$; bottom: $|\delta| = 0.34$; littoral: $|\delta| = 0.79$), and the bottom layer also exhibited a substantial difference relative to the littoral zone ($|\delta| = 0.80$). For *S. lauta*, both the surface and middle-water layers demonstrated large effect sizes when compared to other sampling depths ($|\delta| > 0.50$).

4. Discussion

In this study, we conducted a metabarcoding analysis with mini-barcode primers to assess the effectiveness of environmental DNA (eDNA) in detecting freshwater bivalves in lentic ecosystems and to investigate their spatial distribution patterns. Laboratory tank experiments and field validations confirmed that the primers effectively amplified the

mitochondrial 16S rDNA of freshwater bivalves, successfully detecting multiple Unionidae taxa in lake water samples. Comparative analyses across four lakes revealed that eDNA detection patterns for Unionidae were more significantly influenced by sampling location than by sampling season. Notably, substantial differences in total read numbers were observed between littoral zones and central lake areas, while no statistically significant differences were found among surface, middle, and bottom layers within the central zones of individual lakes. The two dominant species, *Cristaria plicata* and *Sinanodonta lauta*, were consistently detected across all study lakes but displayed distinct spatial read distribution patterns based on sampling location. Overall, these findings suggest that the distribution of freshwater bivalve eDNA in lake water bodies is primarily structured by spatial factors.

The spatial differences in Unionidae eDNA distribution observed in lake environments are likely closely linked to the physical and hydrodynamic characteristics of the water body. Unlike rivers, lakes typically have low flow velocities and prolonged water residence times in autumn and winter. These conditions allow for the stable accumulation of both externally introduced materials and substances released within the water body [22–24]. Consequently, eDNA is more likely to diffuse and mix in the deeper central zones than in littoral areas, maintaining vertical distributions [25,26]. This study found higher total Unionidae read numbers in central lake areas compared to littoral zones, suggesting that the central zones function as hydraulically homogeneous mixing regions that promote stable eDNA distribution.

In addition to read abundance patterns, spatial variation in ASV richness was also observed among sampling positions. In this study, ASV richness represents detection-based diversity within the eDNA dataset rather than a direct measure of true biodiversity or population structure. Therefore, differences in ASV richness should be interpreted as relative variation in eDNA detection patterns across sampling positions, reflecting heterogeneity in eDNA sources and accumulation rather than actual differences in population size.

Interestingly, the lack of statistically significant differences in total Unionidae read numbers among the surface, middle, and bottom layers within the central zones likely indicates well-mixed water column conditions at the time of sampling [27–29]. During autumn and winter, thermal stratification in lakes is often weakened or disrupted, leading to increased vertical mixing [30,31]. Under these hydrodynamic conditions, particulate materials like eDNA are less likely to accumulate in specific depth layers, resulting in a more uniform distribution throughout the water column [32]. Therefore, the similarity in eDNA distributions among depth layers observed in this study is likely due to the physical mixing state of the lake water during the sampling period.

C. plicata and *S. lauta* were consistently detected across all lakes, but their spatial read distribution patterns varied significantly among sampling locations. These variations likely do not represent true population distributions; rather, they may be influenced by species-specific biological traits and life-history characteristics that affect eDNA release and persistence differently [33–35].

At this stage, it is important to note that species-specific differences in eDNA read abundance reflect relative detection signals and proportional contributions within the eDNA pool, rather than absolute biomass or population density. Read abundance is influenced by multiple biological and physical processes, including species-specific eDNA shedding, degradation, transport, and mixing within the water column. Therefore, the observed spatial patterns should be interpreted as relative differences in eDNA signal distribution rather than direct representations of organism abundance.

C. plicata typically prefers deeper benthic habitats, which may enhance both the frequency of eDNA release and its persistence in the deeper central zones of lakes [11].

Supporting this idea, higher read numbers of *C. plicata* were found in the middle and bottom layers of the central lake areas. In lentic systems with long water residence times, eDNA from bottom sediments can accumulate locally or be transported upward into the mid-water layer through vertical mixing processes [36,37]. Therefore, the observed spatial distribution pattern for *C. plicata* likely results from the interplay between its benthic life-style and physical mixing processes within the lake's water column.

In contrast, *S. lauta* is often found in relatively shallow waters and nearshore environments [38]. This may account for the higher frequency of its eDNA detected in surface layers and littoral zones in this study. Littoral areas are marked by frequent sediment disturbance, the presence of aquatic macrophytes, and localized hydrodynamic variability, all of which can lead to temporary concentrations of species-specific eDNA signals [39,40]. As a result, the spatial distribution of *S. lauta* eDNA is likely influenced by a combination of species-specific habitat preferences and fine-scale environmental variations in littoral zones. However, these species-specific spatial patterns should be interpreted with caution, as eDNA detections are indirect observations that do not accurately quantify organism abundance or precise habitat occupancy. Since eDNA signals are affected by factors such as organismal activity, shedding rates, degradation processes, and transport and mixing within the water body [41–43], the observed distribution patterns should be viewed as the result of complex interactions between biological and physical processes, rather than direct representations of population structure [44–46].

Taken together, the observed species-specific differences in read abundance should be interpreted as relative eDNA signal distributions rather than direct indicators of habitat occupancy or organism abundance. Such patterns are shaped by a combination of biological factors (e.g., species-specific eDNA release and degradation) and physical processes (e.g., hydrodynamic transport and mixing) operating within the lake environment. Therefore, the contrasting spatial and seasonal distributions observed between *Cristaria plicata* and *Sinanodonta lauta* are best understood as emergent outcomes of these interacting processes, rather than direct reflections of population density or precise habitat use in the lake.

In this study, the detection patterns of Unionidae eDNA did not show significant seasonal differences, and seasonal effects were minimal even when analyzing specific species. Since sampling was limited to autumn and winter, the study was conducted under relatively stable hydrological and environmental conditions, with little influence from extreme factors such as elevated water temperatures or heavy rainfall. Under these stable conditions, seasonal variation likely had a limited influence on eDNA production, degradation, and transport processes [25,29,47]. Furthermore, eDNA does not necessarily reflect immediate biological activity but may represent accumulated biological signals over time [44,45,48,49]. This characteristic is especially relevant in lentic systems with long water residence times, where eDNA can persist for extended periods, potentially attenuating seasonal signals. Accordingly, even if biological activity differed between autumn and winter, such differences may have been only weakly reflected in eDNA-based detections. Although seasonal differences in read numbers for *S. lauta* were observed, these patterns were not statistically significant and were associated with small effect sizes, suggesting that seasonal variability played a relatively minor role under the conditions examined in this study.

In addition, methodological considerations regarding eDNA filtration should be noted. In this study, eDNA was collected using GF/F filters (0.7 µm), which are widely applied in aquatic eDNA research. Although smaller pore sizes (e.g., ≤0.45 µm) can capture ultra-small extracellular DNA fragments, previous studies have shown that a substantial proportion of aquatic eDNA occurs in particle-associated forms. Considering that this study targeted large-bodied freshwater Unionidae, most detectable eDNA is likely to occur in cellular or particle-bound fractions. For studies focusing on microorganisms or

ultra-fine extracellular DNA, smaller pore sizes may be considered depending on the research objective.

5. Conclusions

Overall, the findings of this study demonstrate that eDNA metabarcoding can effectively detect freshwater Unionidae mussels in lake water columns and reveal clear spatial patterns in eDNA signals within lentic ecosystems. Spatial variation was more pronounced than seasonal variation, with central lake zones showing more homogeneous and stable eDNA signals, while littoral zones exhibited greater heterogeneity influenced by localized environmental conditions. Species-specific differences between *Cristaria plicata* and *Sinanodonta lauta* further highlight the importance of considering both biological traits and physical mixing processes when interpreting eDNA distributions. These findings provide a practical framework for interpreting Unionidae eDNA signals in lakes and offer empirical guidance for designing spatially informed eDNA sampling strategies in lentic environments.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/biology15040338/s1>. Table S1: Geographic information and classification of lake and reservoir sampling sites used for Unionidae eDNA analysis. This table summarizes the names, size categories (medium or small), site codes, and geographic coordinates (latitude and longitude) of the lakes and reservoirs surveyed in this study. For Unmun Lake, two independent sampling sites (UN1 and UN2) were established, whereas a single representative site was selected for each small lake or reservoir. Table S2: Number of Unionidae amplicon sequence variants (ASVs) detected across seasons, species, and sampling locations. The table presents the number of ASVs identified by eDNA metabarcoding according to sampling season (autumn and winter), Unionidae taxa, and sampling positions (littoral zone, surface, middle, and bottom layers) across all study sites. Values represent pooled ASV counts for each category, and total ASV numbers are provided for each grouping and site. Figure S1: Original, uncropped agarose gel electrophoresis images for the CJs and GUn samples. Each gel was run independently and includes both target Unionidae amplicons and additional non-relevant samples that were excluded from Figure 3 for clarity. Red dashed boxes indicate the regions corresponding to the cropped gel images shown in Figure 3. The uncropped images are provided to ensure transparency of gel image processing.

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