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RESEARCH

Seasonal variation in longitudinal connectivity for fish community in the Hotancheon from the Geum River, as assessed by environmental DNA metabarcoding

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Background: Longitudinal connectivity in river systems strongly affects biological components related to ecosystem functioning, thereby playing an important role in shaping local biodiversity and ecosystem health. Environmental DNA (eDNA)-based metabarcoding has an advantage of enabling to sensitively diagnose the presence/absence of species, becoming an efficient/effective approach for studying the community structure of ecosystems. However, little attention has been paid to eDNA-based biomonitoring for river systems, particularly for assessing the river longitudinal connectivity. In this study, by using eDNA we analyzed and compared species diversity and composition among artificial barriers to assess the longitudinal connectivity of the fish community along down-, mid- and upstream in the Hotancheon from the Geum River basin. Moreover, we investigated temporal variation in eDNA fish community structure and species diversity according to season.

Results: The results of species detected between eDNA and conventional surveys revealed higher sensitivity for eDNA and 61% of species (23/38) detected in both methods. The results showed that eDNA-based fish community structure differs from down-, mid- and upstream, and species diversity decreased from down to upstream regardless of season. We found that there was generally higher species diversity at the study sites in spring (a total number of species across the sites [n] = 29) than in autumn (n = 27). Nonmetric multidimensional scaling and heatmap analyses further suggest that there was a tendency for community clusters to form in the down-, mid- and upstream, and seasonal variation in the community structure also existed for the sites. Dominant species in the Hotancheon was *Rhynchocypris oxycephalus* (26.07%) regardless of season, and subdominant species was *Nipponocypris koreanus* (16.50%) in spring and *Odontobutis platycephala* (15.73%) in autumn. Artificial barriers appeared to negatively affect the connectivity of some fish species of high mobility.

Conclusions: This study attempts to establish a biological monitoring system by highlighting the versatility and power of eDNA metabarcoding in monitoring native fish community and further evaluating the longitudinal connectivity of river ecosystems. The results of this study suggest that eDNA can be applied to identify fish community structure and species diversity in river systems, although some shortcomings remain still need to be resolved.

Keywords: artificial barrier, bio-monitoring, conventional survey, eDNA metabarcoding, freshwater fish community, longitudinal connectivity, seasonal variation, species diversity

Introduction

Longitudinal dimension of the river or stream ecosystem from headwaters to mouth may present a continuous or discontinuous gradient of physical conditions, such as water and solutes, that may strongly affect the biological

strategies and river ecosystem dynamics, functioning and services (Allen et al. 2020; Vannote et al. 1980; Wohl 2019). River connectivity is referred to as the degree to which matter and organisms can move among spatially or physically defined units, such as channel and hyporheic zones. It consists of three types of spatial descriptions, including longitudinal, which is from upstream to downstream, lateral and also vertical (Federal Interagency Stream Restoration Working Group 2001; Vannote et al. 1980). Longitudinal connectivity is also involved with movements of water, sediment, wood, solutes and also animals, indicating that it is comprised of physical, chemical and biological connectivity (Leibowitz et al. 2018). There can be longitudinally well connected and wetted river network, disconnected dry river network or a combination of both, which also varies in time or season. The river longitudinal continuum or longitudinal connectivity is regulated largely by fluvial geomorphic processes and strongly affect the following biological components related to ecosystem functioning, such as energy input, organic matter transport, use by macroinvertebrates feeding groups and distribution of freshwater fish species (Consuegra et al. 2021; Vannote et al. 1980). Human made artificial barriers, such as weir, barrage and hydropower dams deteriorate the longitudinal connectivity in river systems by bring about discontinuities in the community structure. Fish ladder or fishway can help to improve the connectivity for the fish community. Assessing the longitudinal connectivity is therefore critical to understand the current status of biodiversity and the ecosystem health of river systems.

Environmental DNA (eDNA) has now become an useful and powerful approach to assess biodiversity for aquatic ecosystems (Pikitch 2018; Ruppert et al. 2019). eDNA is extracellular DNAs in environments, such as sediment, water, snow or air, which are released from skin, mucous, saliva, sperm, and secretions of living organisms (Bohmann et al. 2014). eDNA can provide an efficient and also effective approach for biomonitoring and ecosystem assessments, as it has several advantages, such as high sensitivity, and high feasibility, high reproducibility and also cost effectiveness (Pikitch 2018). Therefore, eDNA has proven to great potential in biodiversity monitoring for the community structure and species diversity in whole freshwater ecosystems (Shaw et al. 2016; Song et al. 2019). Moreover, eDNA enables to detect endangered or rare species of very small populations (Itakura et al. 2019), and also capture invasive species which are expanding their geographic distribution in situ (Jung et al. 2022; Kang et al. 2023). For freshwater fishes, mitochondrial DNA 12S rRNA (MiFish universal primer) has been developed and utilized as a genetic marker for identifying the species (Miya et al. 2015). While a handful of eDNA-based studies of river longitudinal connectivity for freshwater fishes have been undertaken, only little attention has been paid to considering the impacts of artificial barriers on the river connectivity in different seasons (Consuegra et al. 2021; Yamanaka and Minamoto 2016).

In the present study, we applied eDNA method for assessing the longitudinal connectivity of fish community from down-, mid- to up-streams in the Hotancheon River from the Geum River drainage by exploring changes in the community structure, species composition and species diversity among fourteen sites along the river stream. We also compared the results of species identified between eDNA and conventional surveys (e.g., direct field sampling using nets). We have three specific objectives in this study. First, we investigated the fish community structure and species diversity along the Hotancheon River using mitochondrial DNA 12S rRNA (Miya et al. 2015)-based eDNA metabarcoding analysis. Second, we estimated and compared species composition and diversity among the communities separated by artificial barriers such as weir, barrage and fish ladder or fishway to determine the potential impacts of the physical barriers to the river connectivity. By doing so, we were able to assess the longitudinal connectivity of the fish community along down-, mid-, and up-stream by identifying stretches of physical barriers to dispersal, particularly for migratory fishes and species of high mobility.

Materials and Methods

Study sites of eDNA sample collection and field conventional survey

The eDNA water samples were collected from 14 sites, including 4 samples from downstream, 4 midstream, 5 upstream, and 1 reservoir along the Hotancheon River in the Geum River basin in April (spring) and September (autumn) 2022 (Fig. 1, Table 1). There were 11 artificial barriers (sites S2, S3, S4, S5, S6, S8, A3, S10, S11, S12, T2) present along the Hotancheon River, and baffle fishways existed at sites S2, S4, and S5. The field conventional surveys of freshwater fish community were conducted in a quantitative manner at the 10 of 14 sites (S1, S2, S3, S4, S5, S6, S8, S10, S11, and S12), using cast nets (mesh 7 × 7 mm) and kick nets (mesh 4 × 4 mm). The cast net survey was carried out 10 times and the kick net survey was performed for 30 minutes at each of the sites.

Water sampling and filtering

For the eDNA samples, two liter of water was obtained at each station from the water surface using plastic bottles. The bottles were immediately stored an icebox (< 4°C) and transferred to the Molecular Ecology and Evolution Laboratory at the Sangji University. In order to prevent contamination between samples during the eDNA water sampling, water was sampled after arriving at the survey site and

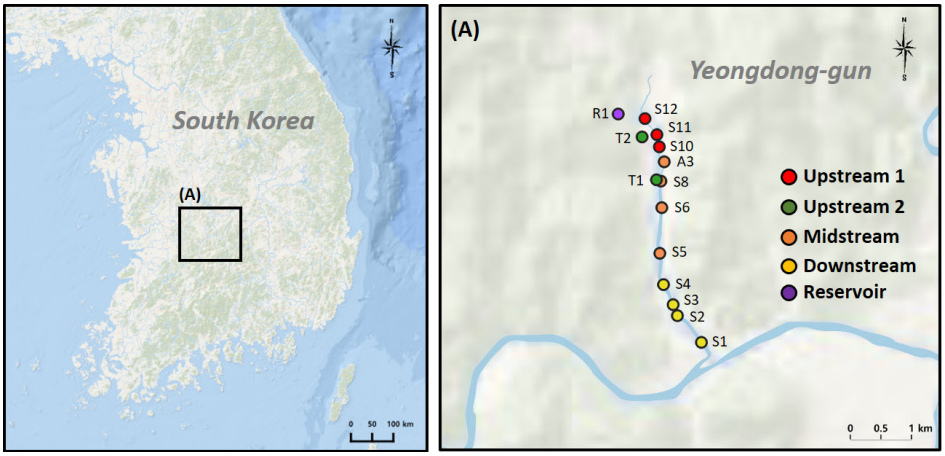

Fig. 1 A map showing 14 collection sites of the eDNA water samples in the Hotancheon River from the Geum River in the Republic of Korea. Artificial barriers are present at every site except for the S1 locality (see Table 1).

Table 1 Physico-chemical characteristics at 14 sampling sites in the Hotancheon River in the Geum River basin in the Republic of Korea

Site	Type	GPS location	Artificial barrier	Spring (2022.04)				Autumn (2022.09)			
				Water temperature (°C)	Water flow rate (ft/s)	Water depth (cm)	Salinity (psu)	Water temperature (°C)	Water flow rate (ft/s)	Water depth (cm)	Salinity (psu)
S1	Down	36°07'57.1"N 127°38'26.8"E	Absence	15.56	0.2	10.1	0.04	21.83	< 0.1	55.0	0.03
S2	Down	36°08'11.2"N 127°38'11.1"E	Presence (fishway)	15.77	0.3	13.0	0.04	21.96	0.2	27.0	0.03
S3	Down	36°08'17.0"N 127°38'08.3"E	Presence	14.11	0.2	11.5	0.04	21.86	0.1	24.0	0.03
S4	Down	36°08'27.6"N 127°38'02.0"E	Presence (fishway)	13.77	< 0.1	22.5	0.04	21.83	0.6	32.3	0.03
S5	Mid	36°08'44.1"N 127°37'59.6"E	Presence (fishway)	13.11	0.2	33.0	0.04	21.56	0.5	70.3	0.03
S6	Mid	36°09'08.2"N 127°38'00.9"E	Presence	13.46	0.5	25.0	0.04	22.38	0.2	61.3	0.03
S8	Mid	36°09'22.2"N 127°38'00.3"E	Presence	15.68	0.6	25.5	0.03	22.58	0.7	22.3	0.05
A3	Mid	36°09'32.4"N 127°38'02.5"E	Presence	15.10	0.1	11.0	0.04	-	0.2	27.0	-
S10	Up	36°09'40.3"N 127°37'59.3"E	Presence	13.00	0.1	14.0	0.04	21.06	0.1	34.0	0.05
S11	Up	36°09'46.7"N 127°37'57.7"E	Presence	13.79	0.2	18.0	0.06	21.12	0.3	30.2	0.05
S12	Up	36°09'55.2"N 127°37'49.9"E	Presence	14.83	0.1	12.0	0.07	21.02	0.1	36.3	0.05
T1	Up	36°09'22.9"N 127°37'57.4"E	Presence	14.13	0.2	15.5	0.03	-	0.3	27.7	-
T2	Up	36°09'45.5"N 127°37'48.0"E	Presence	16.99	0.1	24.5	0.02	-	0.5	18.5	-
R1	Reservoir	36°09'57.6"N 127°37'32.4"E	Absence	18.26	< 0.1	41.0	0.02	21.53	< 0.1	43.0	0.02

Artificial barriers include weir, barrage and fish ladder/fishway (fishway indicated otherwise weir/barrage).
GPS: global positioning system; S: site; A: additional site; T: tributary; R: reservoir.

cleaning the equipment to remove dirt, gravel, dust, and etc. Physico-chemical characteristics of all the sites, such as water temperature, pH, DO, salinity, and ORP were measured using Hanna Multi parameters (HI98194), and flow velocity and water depth were measured using Water Velocity Meter (Fp1111) (Table 1). Water samples collected at each of the 14 locations were filtered twice, 500 mL at each location, using a 0.45 μm membrane filter (GN-6 Metricel®; PALL Life Sciences, Emiliano Zapata, Mexico), a vacuum pump (GAST, Benton Harbor, MI, USA) and a filtering container (NALGENE®; Thermo Fisher Scientific, Waltham, MD, USA). The filtration system was cleaned

with 10% commercial bleach containing sodium hypochlorite to prevent cross-contamination. The filtered membrane filter were put into 2.0 mL tube (Eppendorf, Hamburg, Germany) containing lysis buffer (QIAGEN, Hilden, Germany) and immediately used for DNA extraction.

eDNA extraction

The filtered filter paper was cut into pieces of ~1 mm in size using laboratory scissors disinfected in 10% bleach and then homogenized using a Tissue Lyser II motorized homogenizer (QIAGEN). Genomic DNA was extracted from pre-processed samples using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's protocol. The extracted genomic DNA was measured for concentration using an ND-1000 spectrophotometer (NanoDrop Technologies LLC., Wilmington, DE, USA) and then stored in an ultra-low temperature freezer at −80°C until used for analysis.

MiSeq sequencing and bioinformatics analysis

For NGS-based freshwater fish species analysis of environmental samples collected through field surveys, a library was constructed using the MiFish universal primer (Miya et al. 2015) targeting the 12S rRNA gene (~240 bp) (MiFish-U-F: 5 -GTCGGTAAAACTCGTGCCAGC-3′; MiFish-U-R: 5′-CATAGTGGGGTATCTAATCCCAGTTTG-3′). The following thermal conditions for the primary amplicon production for NGS analysis were applied: initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 15 seconds, and final extension at 72°C for 5 minutes. The amplified PCR product was subjected to electrophoresis and purified using an Accuprep® Gel Purification Kit (Bioneer, Daejeon, Korea) using a 240 bp gel, which was the expected product size. A library was prepared using the Nextera XT index Kit (Illumina, San Diego, CA, USA), and sequence analysis was performed using the Illumina MiSeq platform (2 × 300 bp). The results of base sequence analysis were quality filtered (QV > 20) using CLC genomic Workbench ver 8.0 (CLC Bio LLC., Cambridge, MA, USA), and bioinformatic analysis was performed according to the Miseq data analysis pipeline. The obtained OTUs were described using BLASTn, and those with an identity of 97% or more were classified as species, those with 97% to 90% identity were classified as genus, and those with less than 90% identity were classified as unknown. For ecological interpretation, the relative abundance of the number of OTU reads assigned to each was used to calculate the composition ratio within the community at each site, and data was visualized using Primer-e v7 (PRIMER-E Ltd., Plymouth, UK).

Results

Physico-chemical parameters

The physico-chemical characteristics at the Hotancheon River were followed as average water temperature, 17.85°C (13.00°C–22.58°C), water flow rate, 0.2 m/s (< 0.1–0.7 m/s), water depth, 28.1 cm (10.1–70.3 cm), and salinity, 0.04 psu (0.02–0.07 psu) (Table 1). In spring, the water temperature was 14.83°C (13.00°C–18.26°C), water flow rate 0.2 m/s (< 0.1–0.6 m/s), water depth 19.8 cm (10.1–41.0 cm), and salinity 0.04 psu (0.02–0.07 psu). In autumn, the water temperature was 21.70°C (21.02°C–22.58°C), water flow rate 0.3 m/s (< 0.1–0.7 m/s), water depth 36.4 cm (18.5–70.3 cm), and salinity 0.04 psu (0.02–0.05 psu). It was found that water temperature increases and salinity tends to decrease in autumn relative to spring.

Community structure in the Hotancheon based on eDNA

As results of eDNA metabarcoding analyses, a total of 3,799,513 raw reads were obtained in spring, of which 2,106,600 merged reads (55.44%) were identified, and 1,918,131 merged reads (43.92%) were identified out of a total of 4,366,645 raw reads in autumn (Table 2). Due to the incompleteness of database on the 12S rRNA for Korean freshwater fish species, particularly in the case of Korean endemic species or genetically closely related species groups, species identification was not clear. Therefore, corrections were made by referring to the results of previous conventional surveys (i.e., ecological interpretation). For example, based on the 2016–2018 Survey and Evaluation of Aquatic Ecosystem Health (Water Environment Information System; http://water.nier.go.kr/), *Rhynchocypris kumgangensis* and *Nipponocypris temminckii*, which were found not to inhabit the Hotancheon River, were corrected to related species, *Rhynchocypris oxycephalus* and *Nipponocypris koreanus*, respectively, and *Pseudogobio vaillanti*, an endemic species to China, was not reported to occur in the Republic of Korea, thereby corrected to *Pseudogobio esocinus*.

Results of eDNA metabarcoding analyses showed that a total of five orders, 12 families, and 37 species were present at 14 sites in Hotancheon River (Table 3, Fig. 2). The highest species diversity (three orders, 9 families, 28 species) was detected in the most downstream S1, whereas the lowest species number was observed in reservoir R1 (three orders, five families, 14 species), upstream S10 (three orders, five families, 15 species) and S12 (two orders, five families, 15 species) (Table 3). Fish species diversity significantly decreased from downstream to upstream sites (Pearson correlation, r = 0.7374, $p < 0.001$) (Fig. 3). The most dominant species in all survey locations in the Hotancheon was *R. oxycephalus* (26.07%), which appeared at all locations except R1, and the subdominant species was *N. koreanus*

Table 2 Results of number of sequence reads of fish community structure at each site based on environmental DNA metabarcoding from 14 sites in the Hotancheon River

		Spring (2022.04)			Autumn (2022.09)		
		Raw reads	Processed merged reads	Total number of species	Raw reads	Processed merged reads	Total number of species
Downstream	S1	268,692	158,708	21	568,157	213,925	21
	S2	204,088	142,611	22	188,498	72,454	18
	S3	181,639	99,821	18	229,097	83,150	14
	S4	200,380	92,520	20	151,763	56,343	12
Midstream	S5	202,993	141,719	20	218,653	108,812	11
	S6	200,671	161,092	14	199,454	102,031	12
	S8	191,221	151,859	15	422,134	233,299	12
	A3	212,646	77,703	16	263,325	124,632	6
Upstream 1	S10	226,886	200,732	14	184,420	71,757	4
	S11	264,850	236,599	15	270,102	131,979	6
	S12	248,342	214,854	12	423,825	145,767	7
Upstream 2	T1	206,777	160,607	16	399,825	204,571	13
	T2	246,823	197,279	15	560,064	264,479	10
Reservoir	R1	194,202	70,496	12	287,328	104,932	6
Total		3,799,513	2,106,600	29	4,366,645	1,918,131	27

S1 downstream → S12 upstream, T1, T2: upstream; R1: reservoir.
S: site; A: additional site; T: tributary; R: reservoir.

(15.31%), which was identified at every site. *Pseudopungtungia nigra*, Endangered Wildlife Class I (NIBR 2019), was detected and confirmed at 5 sites in the downstream (S1–S4) and midstream (S5), but the relative frequency was insignificant (0.01%–1.49%). Seven species were identified at all sites: *Squalidus gracilis majimae*, *P. esocinus*, *N. koreanus*, *Zacco platypus*, *Culter* spp., Gobiidae spp., and *Rhinogobius brunneus* (Table 3).

The results of the community similarity analysis of fish community structure among the sites showed high similarities among the S2, S3, S4, S5, and S6 communities, among the A3, S8, S10, S11, and S12 communities, and also between T1 and T2 communities (Fig. 4). These patterns leaded to the formation of a clustering into the mid-down and up-midstream, and R1 as an independent group that was far from other sites. The midstream S6 showed a high similarity with the relatively distant midstream S5 and the downstream S1–S2, while the upstream T1 and T2 showed a high similarity with the mid-downstream S1–S6, and the midstream A3 and S8 showed a high similarity with the upstream S10–S11.

Comparisons of fish community structures by season

With respect to seasonal variation in species diversity across 14 sites in spring and autumn, there were up to 10 more species that occurred in spring than in autumn (Fig. 5). Dominant species in both spring and autumn was *R. oxycephalus* (18.75%, 33.41%), subdominant species in spring was *N. koreanus* (16.20%), and subdominant species in autumn was *Odontobutis platycephala* (15.73%). The relative frequency of the dominant species, *R. oxycephalus* showed no seasonal differences between spring and autumn (Fig. 6). Species that appeared only in spring were identified as *Anguilla japonica*, *Pseudorasbora parva*, *Pungtungia herzi*, *Hemibarbus labeo*, *Culter* spp., *Misgurnus anguillicaudatus*, *Silurus microdorsalis*, *Liobagrus andersoni*, *Oncorhynchus masou masou*, and *R. brunneus*. Species that occurred only in autumn included *Rhodeus* spp., *Acheilognathus yamatsutae*, *Acheilognathus gracilis*, *Squalidus japonicus coreanus*, *Pseudobagrus fulvidraco*, *Lepomis macrochirus*, and *Channa argus* (Table 4). In spring, the lowest number of species (n = 12) was identified at the uppermost S12 and reservoir R1, and the highest number of species (n = 22) was identified at downstream S2. In autumn, the least number of species (n = 4) was identified at midstream S10, and the highest number of species (n = 21) was identified at lower S1 (Fig. 5). The eDNA-based fish species diversity increased from upstream to downstream regardless of season, and the upstream S10 to S12 sites showed relatively lower species diversity than other sites (Fig. 5). In spring, migratory fish species were detected: *A. japonica* (catadromous; 0.54%) was confirmed only at the downstream S3, and *O. masou masou* (anadromous) was confirmed at the downstream S4 (0.15%), midstream S8 (0.47%), and upstream T1 (0.59%) (Table 4). Among the seven species identified at all the sampling sites, patterns of change in relative frequency were confirmed for *N. koreanus* and *Z. platypus*, which are highly mobile due to their high swimming ability, and *R. brunneus*, which can migrate to upstream when they are juveniles. *Nipponocypris koreanus* showed an apparently low frequency of its appearance in upstream S10–S12, and *Z. platypus* showed a sharply low frequency of appearance in

Table 3 Results of relative composition (relative abundance of sequence reads) within fish community structure based on environmental DNA metabarcoding from 14 sites in the Hotancheon River

Species detected	S1	S2	S3	S4	S5	S6	S8	A3	S10	S11	S12	T1	T2	R1	Total
Order Anguilliformes															
Family Anguillidae															
Anguilla japonica			0.27												0.02
Order Cypriniformes															
Family Cyprinidae															
Carassius auratus	0.99	0.06	0.31	1.21			0.08	0.13	0.03	0.32	0.64	1.05	0.15	3.21	0.58
Rhodeus spp.		0.26													0.02
Acheilognathus lanceolatus	4.05	0.12			0.08										0.30
Acheilognathus koreensis[a]	14.80	7.10	21.01	22.08	22.81	33.26	3.72	0.22		0.11		5.58	0.19		9.35
Acheilognathus yamatsutae[a]						0.07									0.01
Acheilognathus gracilis													0.06		< 0.01
Pseudorasbora parva			0.17	0.47	0.29		0.31	0.05	0.25	0.25	0.65	0.29		1.47	0.30
Pungtungia herzi	0.03		0.11	0.10	0.03								0.02	0.18	0.03
Pseudopungtungia nigra[a,c]	1.49	1.03	0.48	0.90	0.01										0.28
Coreoleuciscus splendidus[a]	1.20	0.22													0.10
Squalidus gracilis majimae[a]	0.87	2.59	1.52	2.57	1.40	0.24	0.91	0.79	0.90	0.37	0.26	0.07	0.66	0.90	1.00
Squalidus japonicus coreanus[a]		0.10				0.09									0.01
Hemibarbus labeo	0.67	0.59	0.06	0.05	0.36	0.16		0.34	0.11	0.03	0.02	0.16	0.11		0.19
Pseudogobio esocinus	0.82	0.54	0.11	0.76	1.28	1.53	0.34	0.05	0.15	0.04	0.14	1.63	0.03	0.07	0.53
Microphysogobio yaluensis[a]	0.48	0.10			0.06										0.05
Rhynchocypris oxycephalus	7.45	10.59	12.38	12.81	13.59	17.54	41.04	45.81	51.49	64.39	64.85	13.79	9.31		26.07
Nipponocypris koreanus[a]	13.28	12.56	25.41	24.84	16.84	19.15	20.88	19.02	0.20	0.30	0.08	24.61	29.64	7.48	15.31
Zacco platypus	9.86	1.07	1.68	4.46	0.42	0.38	4.33	3.32	5.26	0.80	1.25	0.47	0.10	8.93	3.02
Opsariichthys uncirostris amurensis	0.12	0.17			0.07	0.04	0.06	0.11	0.02	0.14	0.28	0.20	0.05		0.09
Culter spp.	1.65	31.60	2.51	3.41	15.18	2.42	3.87	8.13	10.67	5.51	3.90	2.88	6.99	4.00	7.34
Family Cobitidae															
Misgurnus anguillicaudatus	1.87	0.31	0.74	0.86	0.82	0.42	2.13	0.74	3.09	1.40	4.86			4.03	1.52
Iksookimia koreensis[a]	9.58	6.12	6.03	4.52	8.63	7.16	4.36					7.45	9.73		4.54
Order Siluriformes															
Family Bagridae															
Pseudobagrus fulvidraco	0.10											0.15	0.26	0.20	0.05
Pseudobagrus koreanus[a]	0.38	0.73	0.35	0.65	0.04	0.78	0.79	0.04	0.13			4.32	1.79	0.39	0.74

Table 3 Continued

Species detected	S1	S2	S3	S4	S5	S6	S8	A3	S10	S11	S12	T1	T2	R1	Total
Family Siluridae															
Silurus microdorsalis[a]		0.10		0.14								0.19			0.03
Family Amblycipitidae															
Liobagrus andersoni[a]	0.06														< 0.01
Order Salmoniformes															
Family Salmonidae															
Oncorhynchus masou masou				0.08			0.23					0.30			0.04
Order Perciformes															
Family Centropomidae															
Siniperca scherzeri										0.10		0.17			0.02
Coreoperca herzi[a]	2.65	1.21	2.69	1.76	2.89	2.79	0.96					4.21	3.03	25.94	3.44
Family Centrachidae															
Lepomis macrochirus[b]	1.54				0.41		0.54					9.33			0.84
Micropterus salmoides[b]	0.07	0.56		0.24	0.24			0.05		0.02	0.20				0.10
Family Odontobutidae															
Odontobutis platycephala[a]	6.36	6.89	14.47	14.07	11.56	9.17	13.36	19.48	26.60	23.80	21.37	19.07	19.63		14.70
Odontobutis interrupta[a]	16.51	12.13	6.23	3.52		0.36									2.77
Family Gobiidae															
Gobiidae spp.	0.24	1.94	2.88	0.15	2.62	4.26	1.46	0.88	0.39	2.16	1.31	3.58	17.72	36.99	5.47
Rhinogobius brunneus	1.10	1.29	0.60	0.35	0.36	0.16	0.62	0.83	0.07	0.27	0.19	0.49	0.51	6.21	0.98
Family Channidae															
Channa argus	1.77														0.13
Number of family	9	9	8	10	8	7	9	6	5	6	5	9	6	5	13
Number of species	28	26	21	23	23	19	19	17	15	17	15	23	19	14	37

S1 downstream → S12 upstream, T1, T2: upstream; R1: reservoir.
S: site; A: additional site; T: tributary; R: reservoir.
[a]Endemic species of the Republic of Korea, [b]introduced species, [c]endangered species.

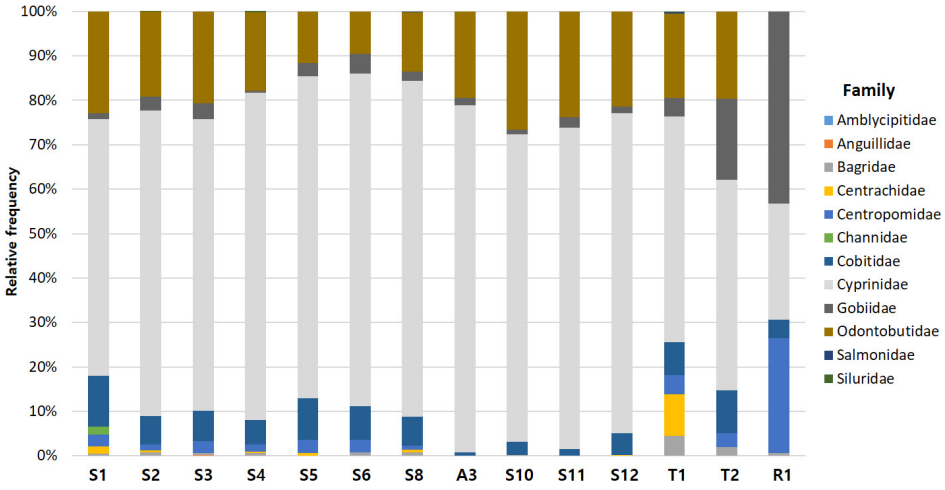

Fig. 2 Proportion of families detected from the 14 sites in Hotancheon River by pooling all datasets using environmental DNA metabarcoding (S1 downstream → S12 upstream, T1, T2: upstream; R1: reservoir).

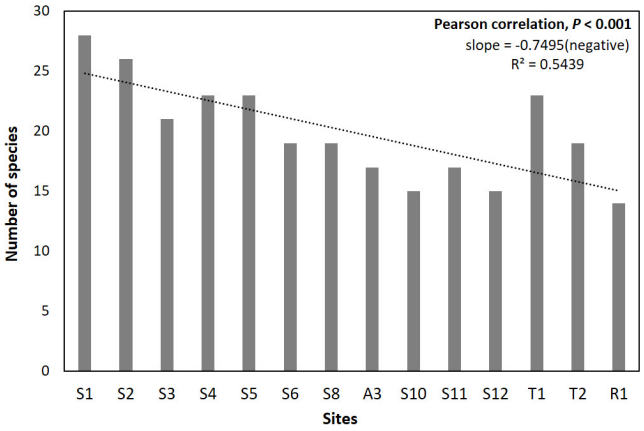

Fig. 3 Trends of changes in species diversity at 14 sites in the Hotancheon River (S1 downstream → S12 upstream, T1, T2: upstream; R1: reservoir) by pooling all datasets.

autumn, and midstream S5–S6 and upstream S11–S12, and it was confirmed that the river network was disconnected after upstream S10 (Fig. 7). *Rhinogobius brunneus*, a benthic fish species, was identified at all locations in spring, but not at all in autumn.

In terms of differences in the fish community structure according to season, there was a difference between the fish community structure in the upstream and mid-downstream, and spring and autumn seasons formed different groupings (Fig. 8).

Comparisons of species detected between field conventional and eDNA surveys

The fish species detected were compared between the conventional survey and eDNA metabarcoding analysis at the 10 sites in the Hotancheon River (S1, S2, S3, S4, S5, S6, S8, S10, S11, and S12) (Fig. 9). The conventional survey and eDNA analysis for the entire community across season showed seven families and 25 species, and 12 families and 36 species, respectively, suggesting higher sensitivity for eDNA method with respect to the species detection (Fig. 9). A total of 38 species were detected from the combined eDNA and conventional surveys, with 23 species (60.5%) commonly detected by both surveys. The two species (*Rhodeus uyekii*, *Hemibarbus longirostris*) were detected only in conventional surveys (5.3% false negatives), and 13 species (*A. japonica*, *Carassius auratus*, *Rhodeus* spp., *S. japonicus coreanus*, *H. labeo*, *Opsariichthys uncirostris amurensis*, *Culter* spp., *S. microdorsalis*, *L. andersoni*, *O. masou masou*, *Siniperca scherzeri*, Gobiidae spp., and *C. argus*) were detected only in eDNA (34.2% false positives), again suggesting that eDNA was more sensitive in detecting the species.

The spring surveys revealed that a total of 29 species were detected from the combined eDNA and conventional surveys, with 14 species (48.3%) commonly detected by both surveys (Fig. 9). All the 14 species detected in conventional method were observed in eDNA (zero false negatives), although 13 species were only detected in eDNA (44.8% false positives). The autumn surveys revealed that a total of 31 species were detected from the combined eDNA and conventional surveys, with 19 species (61.3%) commonly detected by both surveys (Fig. 9). The six species were only detected in conventional method (19.4% false negatives) and the other six species were observed only in eDNA (19.4% false positives). The number of species detected at each of the 10 sites across the season from the conventional survey ranged from four (S11) to 18 (S1), and those from eDNA ranged from 15 (S10 and S12) to 28 (S1) (Table S1).

Discussion

Fish species diversity and community structure in Hotancheon River based on eDNA

In this study, we investigated eDNA-based fish community structure characteristics in the Hotancheon River from the Geum River basin, one of the central river sys-

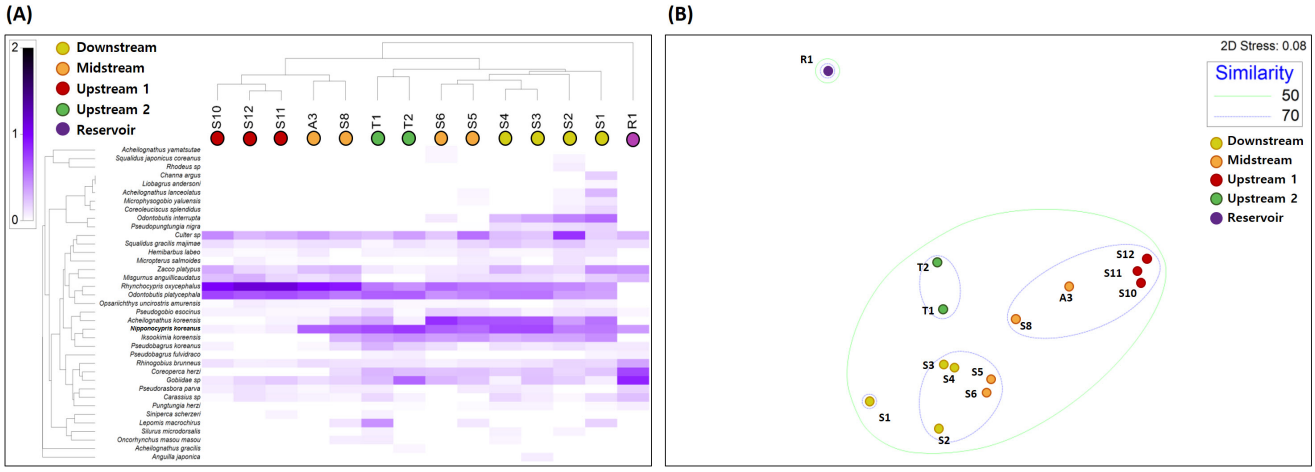

Fig. 4 Results of community similarity analysis of species composition for 14 sites in Hotancheon River by pooling entire dataset. (A) Heat map analysis results. (B) Nonmetric multidimensional scaling (nMDS) plot analysis results.

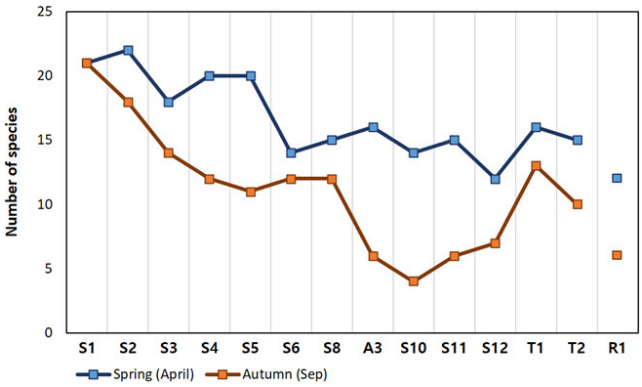

Fig. 5 Comparison of the number of species detected in spring (April) and autumn (September) at 14 sites in Hotancheon River.

tems in the Republic of Korea. We also compared the results of species detection between eDNA and conventional surveys for the 10 study sites. Overall, approximately 61% (23/38) of species for the entire Hotancheon fish community were found in both eDNA and direct field surveys (Fig. 9), indicative of a relatively high match rate. The eDNA analysis allows for detecting more species (eDNA vs. conventional; 36 species vs. 25), suggesting the higher sensitivity for eDNA method for the species detection (Choi et al. 2023. In revision; Shaw et al. 2016; Song et al. 2019).

A total of 37 species from five orders and 12 families were detected as shown by the eDNA analysis. Among them, the family Cyprinidae was detected as the most common taxa with 20 species, which is a general ecological feature of Korean rivers flowing into the West-South Sea and matches well with the freshwater fish fauna of Korean rivers (Jeon 1980; Lee et al. 2012). Fish species diversity tends to decrease from downstream to upstream, showing the consistent pattern as observed in other river systems (Hur et al. 2011; Kim and Lee 2018). Given *L. macrochirus* and *M. salmoides*, which are invasive species, have been identified, and there are endangered species (*P. nigra*) occurring in the Hotancheon River, it would be important to manage the invasive populations in accordance with the manual for removal of ecosystem disturbance species to protect the endangered fish species (Heo et al. 2016). Although migratory fish species such as *A. japonica* and *O. masou masou* were not previously identified in the survey area based on 4th National Natural Environment Survey (2017) and River Aquatic Ecosystem Health Assessment (2018, 2021), their eDNAs were detected in this study. It is hypothesized that the eDNAs of these migratory fishes exist in water-environments, due to the introduction of artificially released fish or water flowing out from a nearby fish farm, as the Hotancheon River is physically isolated completely from the sea (i.e., landlocked), making it impossible for migratory fish species to occur unless artificial or human-induced impacts present (Yoon et al. 2018).

The Hotancheon River fish community was divided into three groups (mid-downstream [S1–S6], mid-upstream [A3, S8, S10, S11, S12], and tributaries [T1, T2]) in terms of similarity. In particular, the similarity of the S1–S6 community is believed to be because the midstream S6 is the confluence of two tributaries. The reason for the low similarity with points S8 and A3, which are geographically closely located to S6, is believed to be that the connectivity of fish from S6 to S8 was reduced due to the artificial structure (weir and barrage) located in the S8. Reservoir R1 showed a fish community structure that was different from that of the river, and this was thought to be due to the dam located in the reservoir acting as a barrier, restricting the movement of fish, resulting in forming divergent community structure (Yamanaka and Minamoto 2016).

At 14 sites in the Hotancheon River, there were up to 10 more species appearing in spring than in autumn, and seasonal changes were confirmed. This result is presumed to be due to factors such as spawning events and growth of juveniles, which may increase the rate of DNA release (eDNA shedding rates; Buxton et al. 2017; Maruyama et al.

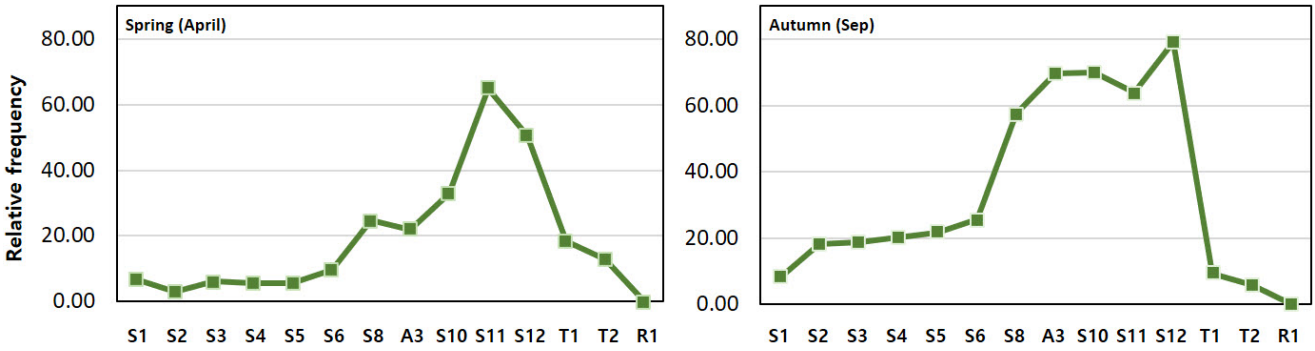

Fig. 6 Relative frequency of *Rhynchocypris oxycephalus*, the dominant species in Hotancheon River, among the sites in spring and autumn.

2014). As a result of comparing the fish community structure by season, there was a difference in similarity between sites, and different groupings were formed in spring and autumn. eDNA shedding can be strongly affected by abiotic factors (flow velocity, water temperature, precipitation, etc.), and high velocity due to high precipitation increases the distance traveled by eDNA but may cause low quality (Barnes et al. 2014). Therefore, it is believed that there is a difference due to physical disturbance of eDNA due to heavy rainfall in August and September (DiBattista et al. 2022). According to DiBattista et al. (2022), fish species richness in eDNA was found to increase with temperature at low rainfall, but not at high rainfall.

Assessment of longitudinal habitat connectivity for fish species

The relative frequencies among sites for two species *N. koreanus* and *Z. platypus*, which are known to have relatively high swimming abilities (i.e., high mobility) and *R. brunneus*, which has low swimming abilities (low mobility) suggest that they could all move to upstreams probably because of using suckers (Misheel et al. 2019; Park et al. 2008; Song and Baek 2005), given they were detected at all sampling sites. *Nipponocypris koreanus* appeared at a sharply low frequency in the upstream S10–S12 sites, and *Z. platypus* was at a low frequency in the midstream S5–S6, and upstream S11–S12. These two species are highly mobile (Misheel et al. 2019; Park et al. 2008), but their population connectivity seems to be restricted after S10 upstream due to artificial structures, and thus they mainly occur in mid-to-downstreams. In addition, since A3 to S12 are mid-upstream and usually have low flow rates, it would be conceivable that *R. oxycephalus*, a species that mainly inhabits upper mountain stream, has a higher frequency than *N. koreanus* and *Z. platypus* (Lee et al. 2017). The rapid decrease in the frequency of *Z. platypus* in the autumn is expected to be the result of lower eDNA shedding and dilution and loss due to the rainy season (Barnes et al. 2014). *Rhinogobius brunneus*, a benthic fish species, was detected at every site in spring, but not at all in autumn. Except for reservoir R1, the relative frequency in spring is relatively low at 0.16%–1.29% and the body size is small, and therefore it would be probable that its absence in autumn is likely due to the influence of the washout phenomenon in the river due to the increase in flow due to the summer heavy rainy season. When the water level increases due to a rainy season, the composition of the community changes as eDNA molecules disperse downstream (Sales et al. 2019; Shogren et al. 2017). Also, the possibility that it was falsely assigned to Gobiidae spp. due to close genetic relationships cannot be ruled out.

Our results suggest that eDNA can be used as a useful tool to identify fish community structure and species diversity in river systems and to evaluate species habitat connectivity, given our observed high match rate (~61%) of species detected between eDNA and conventional surveys. However, we must consider false positives (they are detected but not actually present) and false negatives (they are actually present but not detected) in eDNA results, as moderate and low levels of false positives (34.2%) and false negatives (5.3%) were observed in this study. In the case of the Hotancheon River, which is physically isolated from the sea due to a reservoir, it is highly unlikely that migratory fish species inhabit there. Therefore, the detected *A. japonica* and *O. masou masou* are likely to be false positives. There are three main causes of false positives: (1) contamination of DNA in water samples, (2) inaccurate detection of non-target species during the analysis process, and (3) detection of DNA from dead organisms at the relevant points (Darling and Mahon 2011; Rees et al. 2014, 2015). The solution requires data correction through previous research literature review and interpretation from an ecological perspective (Choi et al. 2023. In revision; Evans et al. 2017). There are two main causes of false negatives: (1) when the DNA of the target species that is actually present in the collected water sample is not detected, and (2) when there is a lack of DNA that can be present in the water sample even though the target species is in its habitat (Darling and Mahon 2011; Rees et al. 2014, 2015). As a solution to false negative results, data errors can be minimized by

Table 4 Results of relative composition (relative abundance) of 12S rRNA sequence reads within the fish community structure by season (spring and autumn) based on environmental DNA metabarcoding at 14 sites in the Hotancheon River

Species detected	Spring															Autumn													
	S1	S2	S3	S4	S5	S6	S8	A3	S10	S11	S12	T1	T2	R1	Total	S1	S2	S3	S4	S5	S6	S8	A3	S10	S11	S12	T1	T2	R1	Total
Order																														
Anguilliformes																														
Family Anguillidae																														
Anguilla japonica			0.01												0.04															
Order																														
Cypriniformes																														
Family Cyprinidae																														
Carassius auratus								< 0.01	< 0.01		0.01		< 0.01	0.06	0.53	0.02	< 0.01	0.01	0.02			< 0.01	< 0.01		0.01	0.01	0.02			0.64
Rhodeus spp.																	0.01													0.04
Acheilognathus lanceolatus	0.01	< 0.01													0.07	0.07	< 0.01			< 0.01										0.54
Acheilognathus koreensis[a]	0.13	0.07	0.30	0.28	0.30	0.56	0.05	< 0.01		< 0.01		0.10	< 0.01		12.81	0.17	0.08	0.12	0.16	0.16	0.11	0.02					0.01			5.89
Acheilognathus yamatsutae[a]																					< 0.01									0.01
Acheilognathus gracilis																												< 0.01		0.01
Pseudorasbora parva			< 0.01	0.01	0.01		0.01	< 0.01	< 0.01	< 0.01	0.01	0.01		0.03	0.60															
Pungtungia herzi	< 0.01		< 0.01	< 0.01	< 0.01							< 0.01	< 0.01	< 0.01	0.07															
Pseudopungtungia nigra[a,c]	0.01	< 0.01	< 0.01	0.02	< 0.01										0.24	0.02	0.02	0.01												0.32
Coreoleuciscus splendidus[a]	< 0.01	< 0.01													0.01	0.02	< 0.01													0.19
Squalidus gracilis majimae[a]	0.01	0.04	0.02	0.03	0.03	< 0.01	0.02	0.02	0.02	0.01	0.01	< 0.01	0.01	0.02	1.60	0.01	0.01	0.01	0.02											0.41
Squalidus japonicus coreanus[a]																	< 0.01				< 0.01									0.03
Hemibarbus labeo	0.01	0.01	< 0.01	< 0.01	0.01	< 0.01		0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		0.38															
Pseudogobio esocinus	0.01	0.01	< 0.01	0.01	< 0.01	0.01	0.01	< 0.01	< 0.01	< 0.01			< 0.01	< 0.01	0.37	0.01	< 0.01	< 0.01	0.01	0.02	0.02	< 0.01				< 0.01	0.03			0.70
Microphysogobio yaluensis[a]		< 0.01			< 0.01										0.02	0.01														0.07

Table 4 Continued

Species detected	Spring															Autumn														
	S1	S2	S3	S4	S5	S6	S8	A3	S10	S11	S12	T1	T2	R1	Total	S1	S2	S3	S4	S5	S6	S8	A3	S10	S11	S12	T1	T2	R1	Total
Rhynchocypris oxycephalus	0.07	0.03	0.06	0.06	0.05	0.10	0.25	0.22	0.33	0.65	0.51	0.18	0.13		18.74	0.08	0.18	0.19	0.20	0.22	0.26	0.57	0.70	0.70	0.64	0.79	0.09	0.06		33.41
Nipponocypris koreanus[a]	0.16	0.08	0.31	0.20	0.10	0.15	0.30	0.33	< 0.01	< 0.01	< 0.01	0.28	0.27	0.08	16.18	0.11	0.17	0.20	0.30	0.23	0.24	0.12	0.05		0.01		0.21	0.32	0.07	14.43
Zacco platypus	0.20	0.02	0.03	0.09	0.01	0.01	0.09	0.07	0.10	0.01	0.02	0.01		0.18	5.96	< 0.01	< 0.01	< 0.01		< 0.01		< 0.01	< 0.01	< 0.01	< 0.01			< 0.01	< 0.01	0.09
Opsariichthys uncirostris amurensis		< 0.01			< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		< 0.01	< 0.01		0.12	< 0.01											0.01				0.06
Culter spp.	0.03	0.63	0.05	0.07	0.30	0.05	0.08	0.16	0.21	0.11	0.08	0.06	0.14	0.08	14.67															
Family Cobitidae																														
Misgurnus anguilli-caudatus	0.04	0.01	0.01	0.02	0.02	0.01	0.04	0.01	0.06	0.03	0.10			0.08	3.04															
Iksookimia koreensis[a]	0.11	0.02	0.03	0.02	0.06	0.07	0.02					0.09	0.14		3.99	0.08	0.10	0.10	0.07	0.11	0.08	0.06					0.06	0.06		5.09
Order Siluriformes																														
Family Bagridae																														
Pseudobagrus fulvidraco																< 0.01											< 0.01	0.01	< 0.01	0.10
Pseudobagrus koreanus[a]	< 0.01	< 0.01		0.01	< 0.01		< 0.01	< 0.01	< 0.01				0.02	< 0.01	0.28	0.01	0.01	0.01	0.01		0.02	0.01					0.09	0.01	0.01	1.20
Family Siluridae																														
Silurus microdorsalis[a]		< 0.01		< 0.01								< 0.01			0.06															
Family Amblycipitidae																														
Liobagrus andersoni[a]	< 0.01														0.01															
Order Salmoniformes																														
Family Salmonidae																														
Oncorhynchus masou masou				< 0.01			< 0.01					0.01			0.09															

Table 4 Continued

Species detected	Spring															Autumn														
	S1	S2	S3	S4	S5	S6	S8	A3	S10	S11	S12	T1	T2	R1	Total	S1	S2	S3	S4	S5	S6	S8	A3	S10	S11	S12	T1	T2	R1	Total
Order Perciformes																														
Family																														
Centropomidae																														
Siniperca scherzeri										< 0.01					0.01												< 0.01			0.02
Coreoperca herzi[a]	0.03	< 0.01	0.01		< 0.01	< 0.01						0.04	0.03	0.34	3.23	0.03	0.02	0.05	0.04	0.05	0.05	0.02					0.04	0.03	0.18	3.65
Family																														
Centrachidae																														
Lepomis macrochirus[b]																0.03				0.01		0.01					0.19			1.69
Micropterus salmoides[b]	< 0.01	0.01		< 0.01	< 0.01			< 0.01		< 0.01	< 0.01				0.17											< 0.01				0.03
Family																														
Odontobutidae																														
Odontobutis platy-cephala[a]	0.07	0.03	0.13	0.15	0.09	0.04	0.12	0.16	0.24	0.17	0.26	0.21	0.24		13.67	0.06	0.11	0.16	0.13	0.14	0.14	0.14	0.23	0.29	0.30	0.16	0.17	0.16		15.73
Odontobutis interrupta[a]	0.09	0.02	0.02	0.02											1.08	0.24	0.23	0.11	0.05		0.01									4.46
Family Gobiidae																														
Gobiidae spp.																< 0.01	0.04	0.06	< 0.01	0.05	0.09	0.03	0.02	0.01	0.04	0.03	0.07	0.35	0.74	10.94
Rhinogobius brunneus	0.02	0.03	0.01	0.01	0.01	< 0.01	0.01	0.02	0.01	0.01	< 0.01	0.01	0.01	0.12	1.96															
Family Channidae																														
Channa argus																0.04														0.25
Number of family	8	8	6	8	7	5	5	6	5	6	5	7	6	5	11	8	6	6	6	6	6	7	3	3	3	4	7	6	4	8
Number of species	21	22	18	20	20	14	15	16	14	15	12	16	15	12	29	21	18	14	12	11	12	12	6	4	6	7	13	10	6	27

S1 downstream → S12 upstream, T1, T2: upstream, R1: reservoir.
[a]Endemic species of the Republic of Korea, [b]introduced species, [c]endangered species.

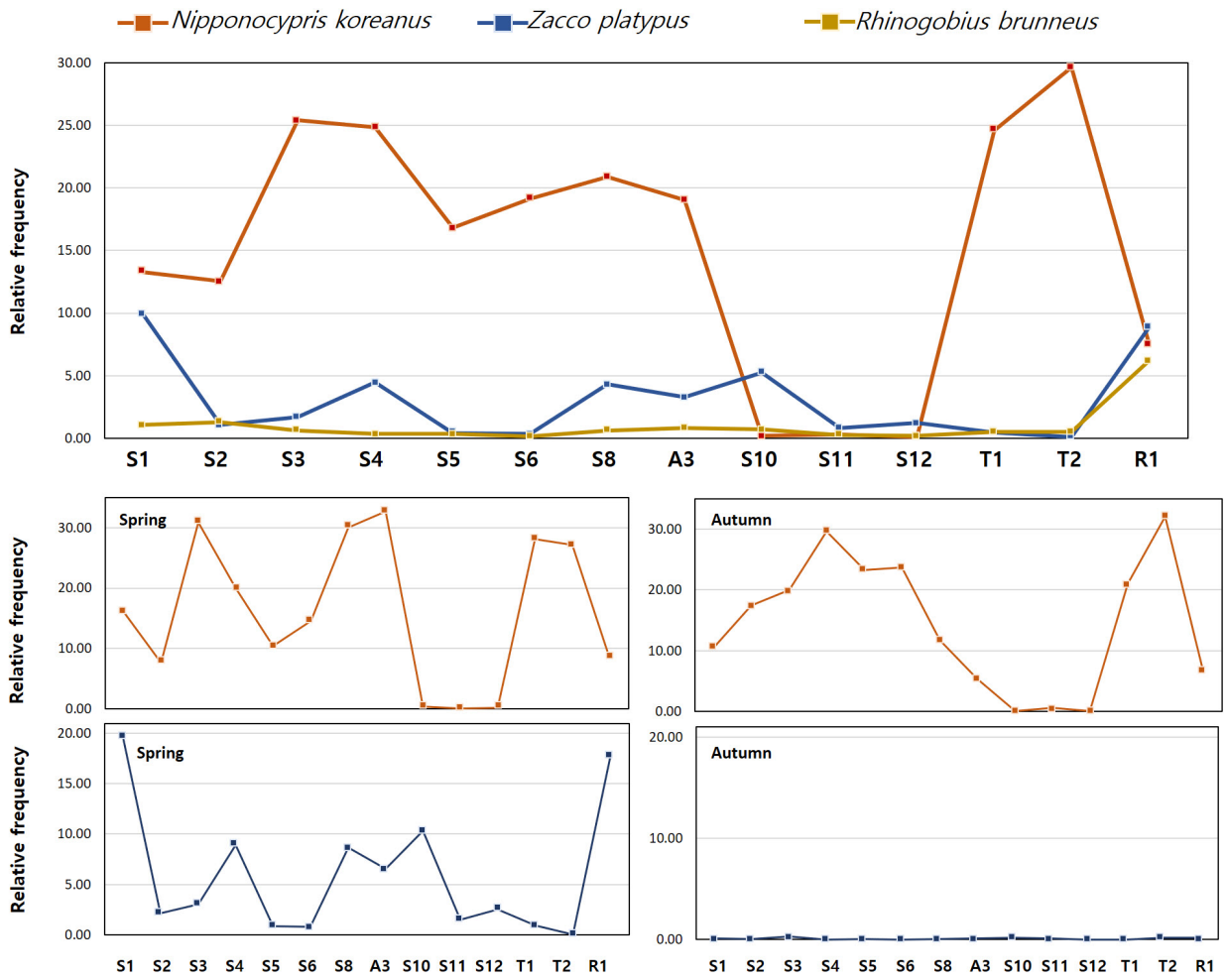

Fig. 7 Changes in relative frequencies of the three dominant species (*Nipponocypris koreanus, Zacco platypus* and *Rhinogobius brunneus*) identified at all 14 sites in Hotancheon River.

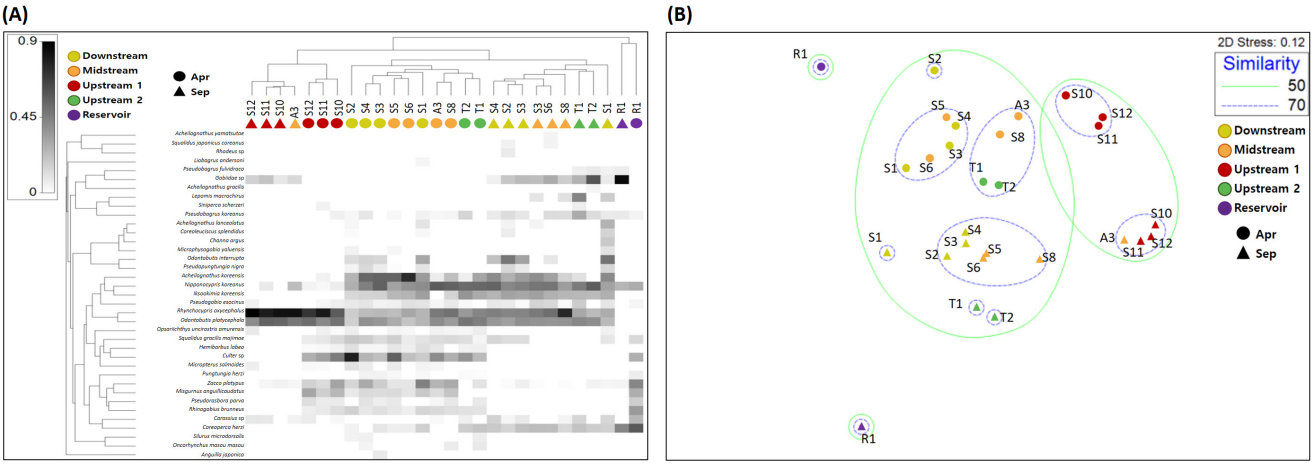

Fig. 8 Results of community similarity analysis by season for the 14 Hotancheon River sites. (A) Heatmap analysis results. (B) Nonmetric multidimensional scaling (nMDS) plot analysis results.

increasing the number of water sample points and samples for eDNA analysis, and it is also considered necessary to build a database of undetected species (Evans et al. 2017). If these problems are corrected, it is believed that the eDNA metabarcoding technique can be applied as a very efficient and practical tool to identify fish community structure and species diversity, and to evaluate species connectivity according to artificial structures.

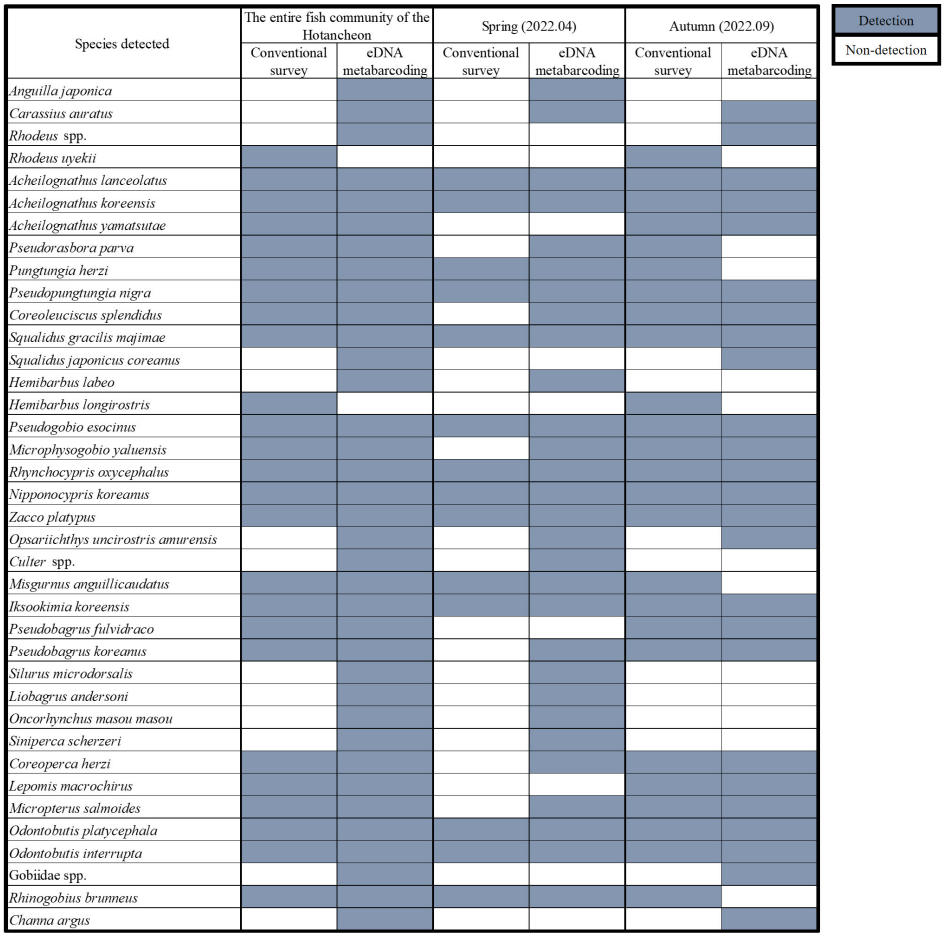

Fig. 9 Comparisons of species detected from the 10 sites in the Hotancheon River by conventional and environmental DNA (eDNA) surveys. The boxes filled in color represent species detected and those not filled (in white) represent species not detected.

Conclusions

This study applied eDNA to analyzing and comparing species diversity and composition for freshwater fish communities between artificial barriers to determine the longitudinal connectivity along the down-, mid-, and upstream in the Hotancheon stream from the Geum River basin in the Republic of Korea. In addition, we examined temporal variation in the eDNA fish community structure and species diversity according to season (spring and autumn). We found that eDNA-based fish community structure was dissimilar between upstream and mid-downstream, and species diversity tend to increase from upstream to downstream regardless of season. With respect to seasonal variation in fish community, the results showed that species diversity was generally higher in spring than in autumn. Nonmetric multidimensional scaling (nMDS) and heatmap analyses further support our findings that there was a tendency for separate clusters to form among the down-, mid-, and upstreams, and seasonal variation also existed by sites. Artificial barriers (e.g., weir) appeared to negatively affect the connectivity of some fish species (e.g., *N. koreanus*, *Z. platypus*) of high mobility. Our results suggest that eDNA can be used as a useful approach to identify fish community structure and species diversity and further assess longitudinal connectivity, given its high sensitivity and also high reliability for species detection. We highlight that this method would greatly contribute to establishing biological monitoring systems, particularly for freshwater fishes.

Supplementary Information

Supplementary information accompanies this paper at https://doi.org/10.5141/jee.23.067.

Table S1 Comparisons of species detected between conventional and eDNA surveys at the 10 study sites in the Hotancheon River.

Abbreviations

eDNA: Environmental DNA

nMDS: Nonmetric multidimensional scaling

Acknowledgements

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Authors' contributions

HJL, KGA, SJK, and DYB contributed to the study conception and

design. YRK, HC, SYB, and SYH contributed to material preparation, data collection and analysis, and interpretation of results. HJL and YRK wrote the first draft and HC, SYB, SYH, KGA, SJK, and DYB commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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