Molecular Monitoring of an Invasive Freshwater Fish, Brown Trout (*Salmo trutta*), Using Real-Time PCR Assay and Environmental Water Samples

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Article

Molecular Monitoring of an Invasive Freshwater Fish, Brown Trout (*Salmo trutta*), Using Real-Time PCR Assay and Environmental Water Samples

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Abstract: *Salmo trutta,* commonly known as brown trout, is an invasive species that has established itself in various regions, including South Korea, where it poses ecological risks to native freshwater fish populations. The Ministry of Environment of South Korea has legally designated *S. trutta* as an invasive species. Thus, to enable natural habitat restoration, *S. trutta* needs to be monitored in South Korea. However, traditional monitoring techniques are associated with several limitations. Therefore, in this study, we aimed to apply a sensitive and specific qPCR assay using primers and a hydrolysis probe specific to the mitochondrial cytochrome *b* gene (*mt-cyb*) of *S. trutta*. Environmental DNA (eDNA) was extracted from river water samples collected downstream of the Soyang Reservoir and around the Uiam Reservoir between January and March 2023. The qPCR assays successfully detected *S. trutta* eDNA in 11 of the 24 samples, with high concentrations found upstream and downstream of the Soyang River. Our results demonstrate the effectiveness of qPCR assay for the detection of *S. trutta* in aquatic environments and highlight its potential for monitoring the spread of this species, especially in areas that are difficult to survey using traditional methods. This molecular approach offers a more efficient tool for *S. trutta* population management, mitigating its impact on native biodiversity.

Keywords: environmental DNA; invasive species; molecular monitoring; *Salmo trutta*

Introduction

Salmon and trout are characterized by elongated and streamlined bodies, enabling fast swimming in both freshwater and marine environments. Most of them are anadromous and their life cycle includes distinct stages: egg, fry, smolt, and adult, with some species displaying remarkable homing abilities and returning to their natal streams for reproduction (Groot and Margolis 1991; Quinn 2007). In South Korea, 11 salmoniform species have been identified (Kim et al. 2005). Among these, three species, *Brachymystax lenok tsinlingensis*, *Oncorhynchus keta*, and *Oncorhynchus masou masou*, are native to South Korea, whereas *Oncorhynchus mykiss* was introduced for aquaculture and recreational fishing. These species play significant roles in Korean riverine ecosystems and are both culturally and commercially important.

Salmo trutta, endemic to most of Europe and commonly referred to as brown trout, is a freshwater migratory species that spawns in riverbeds composed of gravel or sand with fast-flowing, cool, and oxygen-rich waters. This species exhibits a high degree of behavioral variability, with some populations being purely freshwater residents, whereas others are anadromous, known as sea trout, and migrate to the ocean before returning to rivers to spawn. This species is prized for its adaptability, thrives in a wide range of habitats, and is well-known for sport fishing (Elliott 1994; Klemetsen et al 2003).

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Over the past few decades, *S. trutta* has been introduced into many regions outside of its native habitats, including North America, Australia, and parts of Asia, including South Korea, for food consumption and recreational fishing (Townsend 1996; Klemetsen et al. 2003; Behnke 2010; McIntosh et al. 2010; Korsu et al. 2010; Hasegawa 2020; Park et al. 2022). As an aggressive predator and competitor, this alien species outcompetes native fish for food and habitat and preys on native aquatic animals, disrupting the natural aquatic ecosystems; thus, *S. trutta* is a significant ecological threat to several non-native regions (Townsend and Crowl 1991; Klemetsen et al. 2003; McDowall 2003; McHugh and Budy 2006; Korsu et al. 2010; McIntosh et al. 2010; Hasegawa 2020). In addition, *S. trutta* hybridizes with native salmonid species (Verspoor 1988; Meldgaard et al. 2007; Castillo et al. 2008), reducing the genetic integrity and fitness of native populations. Genetic mixing threatens the long-term viability of native species, exacerbating their decline and destabilizing freshwater ecosystems in which these species play critical roles. Owing to the ecological threats exerted by this species, the International Union for Conservation of Nature and Natural Resources (IUCN) has listed *S. trutta* in the "100 of the World's Worst Invasive Alien Species" (Lowe et al. 2000).

Although exact records of its introduction are unavailable, Park et al. (2022) reported that *S. trutta* successfully established a sexually mature population downstream of the Soyang Reservoir in Chuncheon-si, Gangwon-do, South Korea. This area is enclosed by three reservoirs: Soyang Reservoir, which discharges water into the Soyang River and drains into the northeastern Uiam Reservoir; Paro Lake, which is connected to the Bukhan River and drains into the northwestern Uiam Reservoir; and southern Uiam Reservoir, which discharges water into the Han River Basin. As a result, the *S. trutta* population that was introduced into South Korea became landlocked. As a cold-water species, its optimal habitat temperature ranges between 12 and 19 °C; however, it is resilient and survives at temperatures up to 24.7 °C (Molony 2001). During summer, most rivers in South Korea approach 30 °C, limiting the species' ability to establish a wide distribution. Nevertheless, owing to hydroelectric power generation, the multipurpose dam continuously discharges cold water from its mid-layer, creating conditions that allow for *S. trutta* survival downstream.

In 2021, the Ministry of Environment of South Korea legally designated *S. trutta* as an invasive species to prevent further spread and minimize ecological damage through eradication and removal projects (ME 2021; NIE 2023). Owing to its popularity in recreational fishing and ecological adaptability, there are concerns regarding the impact of widespread *S. trutta* release and distribution. Therefore, close monitoring of habitat and potential spread is required, necessitating the urgent need for rapid and effective species identification in new habitats and areas of expansion. However, the current assessment and monitoring of *S. trutta* are still conducted using traditional methods, such as skimming nets, gill nets, or angler-provided rod catch data. Electrofishing, a common method used in freshwater fish surveys, is illegal in South Korea. Given ecological traits of *S. trutta*, including its preference for cold water and long-distance migration, monitoring via traditional methods is challenging. Moreover, these surveys are expensive, labor-intensive, and time-consuming.

Environmental DNA (eDNA)-based real-time PCR (quantitative PCR or qPCR) assays are powerful tools for molecular monitoring of invasive fish species in aquatic ecosystems, as well as for monitoring commercially exploited, rare, or endangered fish species (e.g., Wilcox et al. 2015; Atkinson et al. 2018; Fernandez et al. 2018; Knudsen et al. 2019; Hernandez et al. 2020; Kim et al. 2021). This method involves the collection of water samples from an environment where a species may be present and analysis of the obtained samples for the detection of biological material, such as skin cells, scales, mucus, blood, feces, and gametes, shed by the species. qPCR amplifies DNA sequences unique to the target species, enabling highly sensitive detection even at low population densities. This technique is particularly useful for early detection, tracking the spread, and monitoring the distribution of exotic fish species, such as *S. trutta* (Gustavson et al. 2015; Banks et al. 2016; Carim et al. 2016; Deutschmann et al. 2019; Hernandez et al. 2020).

Therefore, in this study, we aimed to determine the distribution range of *S. trutta* through qPCR analysis of water samples collected from river water downstream of the Soyang Reservoir and around the Uiam Reservoir, where the species is known to inhabit.

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Materials and Methods

Sampling of Fish Specimens and Genomic DNA (gDNA) Extraction

Specimens of *S. trutta* ($n = 2$), the other five salmonid species, i.e., *B. lenok tsinlingensis*, *O. keta*, *O. mykiss*, *Thymallus grubii*, and *Salmo salar*, and 19 common freshwater fish species were collected from rivers or local markets in South Korea. A piece of the salmonid pelvic fin was excised and used for gDNA extraction, as previously described by Asahida et al. (1996). The extracted gDNA was resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and its quantity and quality were assessed using a spectrophotometer (NanoDrop™ One, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Modification of Primers and Probe

All mitochondrial cytochrome *b* gene (*mt-cyb*) sequences of *S. trutta* haplotypes and all reference sequences of mitochondrial genomes of salmoniform species were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/) and aligned using ClustalW in BioEdit 7.2 (Hall 1999). Following comparison of the aligned nucleotide matrices, the forward and reverse primers and hydrolysis probe reported by Carim et al. (2016) were modified, which were used in the qPCR assays. The melting temperature (Tm) and secondary structures of the primers were predicted using the Sequence Manipulation Suite ver. 2 (https://www.bioinformatics.org/sms2/) (Stothard 2000) and optimized prior to oligonucleotide synthesis.

Environmental Water Sampling and eDNA Extraction

Environmental water samples (each 1–2 L) were collected in sterile disposable plastic bottles at a depth of 10 m from eight stations located downstream of the Soyang Reservoir and around the Uiam Reservoir between January and March 2023 (Table 1) in Chuncheon-si, Gangwon-do, South Korea. The samples were vacuum-filtered through glass microfiber filters (Grade GF/F circles, 47 mm; Whatman, Marlborough, MA, USA). Each filter was folded in half four times using uncontaminated forceps, inserted into a 2.0-mL microtube, and placed in a cooled ice box with polyethylene bags containing ice, protected from light exposure. They were then transported directly to the laboratory and stored in at –70 °C until further use. Each filter was broken using a Omni Bead Ruptor 12 Bead Mill Homogenizer (OMNI International, Kennesaw, GA, USA) and eDNA was extracted using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The eDNA was finally eluted in 50 μL of sterile distilled water and immediately stored at –20 °C for further processing.

Table 1. Information on environmental water sampling sites for molecular monitoring of the brown trout, *Salmo trutta*, downstream of the Soyang Reservoir and around the Uiam Reservoir in Chuncheon-si, Gangwon-do, South Korea.

Station	GPS coordinate	Location
St. 1	37°55′37.17″N 127°47′07.03″E	Upstream of the Soyang River, downstream of the Soyang Reservoir
St. 2	37°54′15.93″N 127°42′46.12″E	The Sangjung Island, upstream of the Uiam Reservoir
St. 3	37°52′08.34″N 127°43′26.22″E	The Gongji Stream flowing into the Uiam Reservoir
St. 4	37°54′57.30″N 127°43′05.38″E	Downstream of the Bukhan River
St. 5	37°53′39.78″N 127°41′23.97″E	Small stream flowing into the Uiam Reservoir
St. 6	37°51′08.81″N 127°40′07.78″E	Small stream flowing into the Uiam Reservoir
St. 7	37°51′21.98″N 127°41′09.69″E	Downstream of the Uiam Reservoir
St. 8	37°53′40.60″N 127°43′25.11″E	Downstream of the Soyang River, downstream of the Soyang Reservoir

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qPCR Assay

qPCR amplification was conducted in triplicate (three technical replicates) for each eDNA sample using GoTaq® Probe qPCR Master Mix (Promega, Madison, WI, USA). The reaction mixture (10 µL) contained 1 µL of eDNA as a template, 0.2 µM each of the forward Str-cyb-0297f (5′-CCGAGGACTCTACTATGGT-3′) and reverse Str-cyb-0384r (5′-GGAAGAACGTAGCCCACG-3′) primers, and the hydrolysis probe Str-cyb-0341p (5′-FAM-ATATCGGAGTCGTACTGCTA-MGB-Eclipse-3′), which were synthesized by Macrogen, Inc. (Seoul, South Korea). qPCR was performed using a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific) with the following cycling conditions: an initial activation at 95 °C for 2 min, followed by 50 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 45 s. The gDNAs (20 ng μL^{-1}) extracted from two *S. trutta* specimens were used as positive controls. Sterile distilled water was used as a negative control to monitor contamination during filtration, eDNA extraction, and qPCR analysis.

A synthesized partial DNA fragment of *S. trutta mt-cyb* containing all the binding sites for the forward and reverse primers and the hydrolysis probe was produced by gene synthesis and inserted into a plasmid by Bioneer Inc. (Daejeon, South Korea). The sensitivity of the oligonucleotide primers and the hydrolysis probe was tested against 10-fold serial dilutions of the plasmid DNA (10^9 copies rxn^{-1}) in triplicate for each dilution. The results were used to produce a standard curve for *S. trutta* eDNA. Their specificity was also tested against five other salmonid species and 19 freshwater fish belonging to diverse orders and families that are commonly found in South Korea.

Results

Modified Primers and Probe

In this study, we modified the forward and reverse primers, as well as the hydrolysis probe, previously described by Carim et al. (2016), which were specific to *S. trutta* and designed based on *mt-cyb* sequences (Table 2). The Tm of the forward and reverse primers used in this study (Str-cyb-0297f and Str-cyb-0384r, respectively) were lower than those used by Carim et al. (2016) (Str-cyb-0294f and Str-cyb-0382r, respectively). Both primers used in the present study showed exact matches with all *S. trutta* haplotypes in the GenBank database. Additionally, the hydrolysis probe used in this study (Str-cyb-0341p) had a higher Tm than that used by Carim et al. (2016) (Str-cyb-0345p). The probe matched all *S. trutta* haplotypes in the GenBank database, with a single base-pair mismatch in only one haplotype (GenBank accession number JX960839) reported by Crête-Lafrenière et al. (2012), but multiple base-pair mismatches with other salmoniform species, including the congeneric *Salmo ischchan*, *Salmo obtusirostris*, and *Salmo salar*.

Table 2. Comparison of the forward and reverse primers and hydrolysis probe from Carim et al. (2016) and those modified in this study, which are specific to the brown trout, *Salmo trutta.*

Oligonucleotide name[1]	Sequence (5′ → 3′)[2]	Sequence length (bp)	GC content (%)	Nearest neighbor Tm (°C)	Reference
Forward primer					
Str-cyb-0294f	CGCCCGAGGACTCTACTATGGT	22	59.09	67.87	Carim et al. (2016)
Str-cyb-0297f	CCGAGGACTCTACTATGGT	19	52.63	60.07	This study
Reverse primer					
Str-cyb-0382r	GGAAGAACGTAGCCCACGAA	20	55.00	65.00	Carim et al. (2016)
Str-cyb-0384r	GGAAGAACGTAGCCCACG	18	61.11	62.94	This study
Hydrolysis probe					
Str-cyb-0345p	CGGAGTCGTACTGCTAC	17	58.82	58.83	Carim et al. (2016)
Str-cyb-0341p	ATATCGGAGTCGTACTGCTA	20	45.00	60.01	This study

[1]The oligonucleotides from Carim et al. (2016) were named based on the species name, with the first letter of the genus (S) and the first two letters of the species (tr) for *S. trutta*, followed by the gene name mitochondrial

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cytochrome *b* gene (*mt-cyb*), the relative position of the first base of each oligonucleotide from the start codon of *mt-cyb*, and their direction forward (f) or reverse (r) or function probe (p). [2]The consensus sequences between Carim et al. (2016) and this study are underlined for each primer and hydrolysis probe.

The forward and reverse primers produced a 105-bp amplicon, as predicted using conventional PCR amplification. The hydrolysis probe consisted of a 20-mer oligonucleotide with a fluorophore, fluorescein, covalently attached to the 5′-end, and a quencher, MGB-Eclipse, at the 3′-end. This combination of oligonucleotides was unique to *Salmo trutta* and was not found in other salmoniform species.

Sensitivity and Specificity Tests

qPCR amplification using the designed primers and probe resulted in positive amplification with a quantification cycle (Cq) value of 17.776 against a standard concentration of the gDNA (20 ng μL^{-1}) extracted from *S. trutta*. No background amplification was observed. The sensitivity test was conducted using a serial dilution of plasmid DNA (1–10^9 copies per reaction), in which a partial DNA fragment of *S. trutta mt-cyb* containing all the binding sites for the forward and reverse primers and the hydrolysis probe were inserted. An inverse relationship was observed between the Cq values and plasmid DNA concentrations, with a detection limit of as low as 10^2 copies per reaction of plasmid DNA and a Cq value of 36.717 (Figure 1). Therefore, the Cq value was set as the lowest detection limit to achieve acceptable levels of precision and accuracy in the qPCR assay. This correlation was used to generate the standard curve slope, which produced the following linear regression equation:

$$y = -3.51x + 43.818 \ (r^2 = 0.999, \text{efficiency} = 92.69\%)$$

Linear regression was used to detect and quantify *S. trutta* eDNA in environmental water samples.

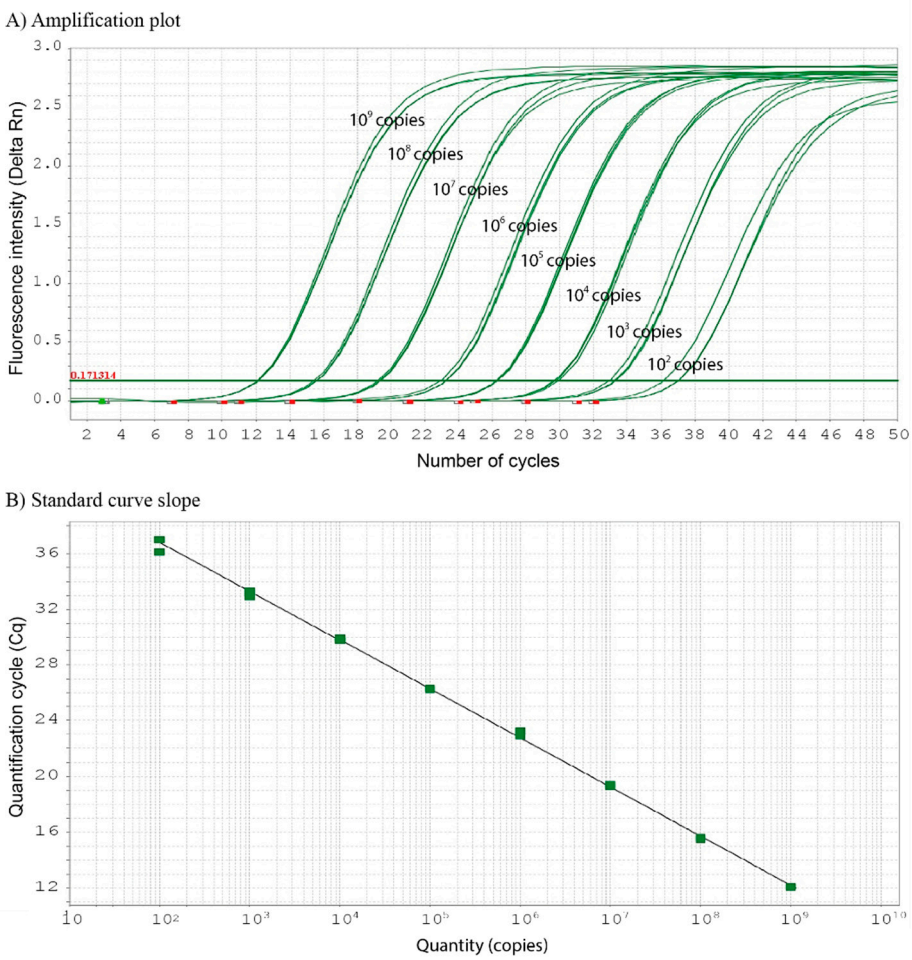

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Figure 1. Amplification plot and standard curve of the real-time PCR assay using the primers and hydrolysis probe specific to brown trout, *Salmo trutta*. A serial dilution of plasmid DNA (1–10^9 copies per reaction) shows a detection limit of as low as 10^2 copies per reaction of plasmid DNA (A), with standard curve slope producing a linear regression (B).

To confirm the specificity of the primers and hydrolysis probe for *S. trutta*, we carried out qPCR amplification of 5 salmonid species (*B. lenok tsinlingensis*, *O. keta*, *O. mykiss*, *T. grubii*, and *S. salar*) and 19 freshwater fishes belonging to diverse orders and families (*Acheilognathus rhombeus*, *Coreoperca herzi*, *Cyprinus carpio*, *Gasterosteus aculeatus*, *Hemiculter eigenmanni*, *Lepomis macrochirus*, *Liobagrus andersoni*, *Misgurnus anguillicaudatus*, *Monopterus albus*, *Mugil cephalus*, *Odontobutis platycephala*, *Orthrias nudus*, *Oryzias latipes*, *Plecoglossus altivelis*, *Repomucenus olidus*, *Rhynchocypris oxycephalus*, *Silurus asotus*, *Tachysurus fulvidraco*, and *Zacco platypus*) that are commonly found in rivers and lakes or local markets of South Korea. Only two *S. trutta* specimens successfully produced positive fluorescence amplifications, whereas the other five salmonid and freshwater fish failed to produce measurable amplifications (Figure 2).

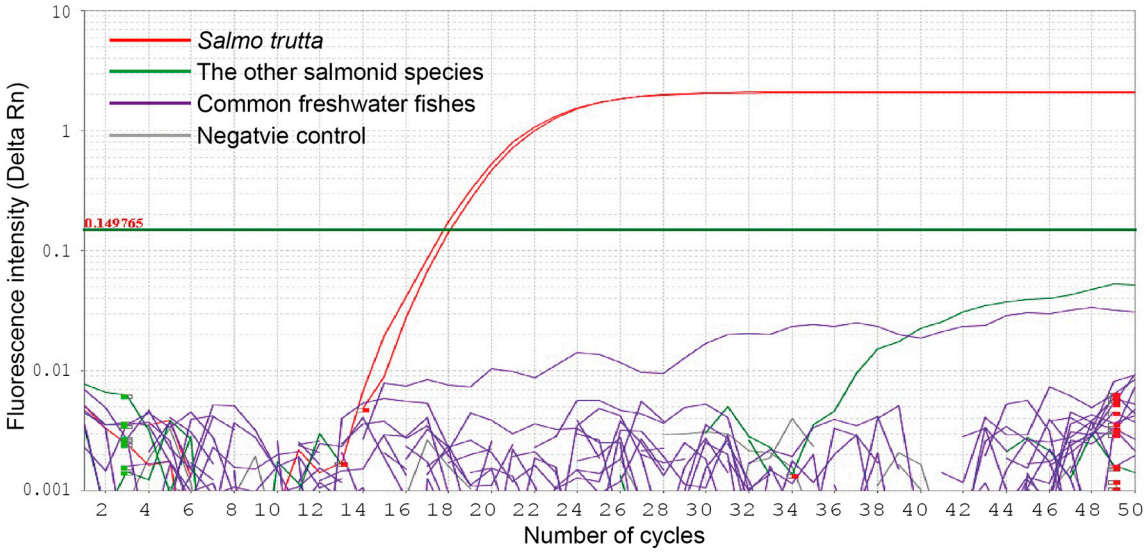

Figure 2. Amplification curve of real-time PCR analysis using the primers and hydrolysis probe specific to brown trout, *Salmo trutta*. Specificity testing was conducted on five salmonid species, *Brachymystax lenok tsinlingensis*, *Oncorhynchus keta*, *Oncorhynchus keta*, *Thymallus grubii*, and *Salmo salar*, as well as two *S. trutta* specimens and 19 freshwater fish species commonly found in South Korean rivers and lakes or local markets.

qPCR Assay of Environmental Water Samples

The *S. trutta*-specific primers and hydrolysis probe used in this study were used to amplify eDNA extracted from the collected environmental water samples (n = 24). Positive amplifications were observed in 11 samples (Stns. 1, 2, and 8 on January 2023 at Stns. 1, 2, 5, and 8 in February 2023, and Stns. 1, 6, 7, and 8 in March 2023), with Cq values ranging from 30.665 to 38.629, corresponding to plasmid copy numbers from 250.6 copies L^{-1} to 132,677.1 copies L^{-1} (Table 3). The highest value, 132,677.1 ± 6,386.3 copies L^{-1}, was observed for St. 1.

S. trutta eDNA was detected in all three replicates in the upstream section of the Soyang River (St. 1), located downstream of the Soyang Reservoir, between January and March 2023 (Figure 3). It was also detected in all three replicates from the downstream section (St. 8) in February 2023 but in only one replicate out of three in January and March. Additionally, it was detected in all three replicates from Sangjung Island (St. 2), located upstream of the Uiam Reservoir, in January and February 2023; however, no *S. trutta* eDNA was detected in March. In contrast, two small streams flowing into the Uiam Reservoir (Stns. Five and 6) possessed *S. trutta* eDNA in only one out of three replicates in February and March, whereas St. 7, located downstream of the Uiam Reservoir, showed

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S. trutta eDNA in only one of the three replicates in March at very low concentrations. However, *S. trutta* eDNA was not detected in the Gongji Stream (St. 3), which flows into the Uiam Reservoir, or in St. 4, which is located downstream of the Bukhan River. Representative samples that showed positive amplifications were further verified via Sanger sequencing to confirm the absence of false-positive results (data not shown).

Table 3. Quantification of environmental DNA from water samples collected downstream of the Soyang Reservoir and around Uiam Reservoir using real-time PCR analysis with primers and a probe specific to brown trout, *Salmo trutta.*

Water sample	Volume (mL)	Quantification cycle (Cq) value[1]			Total volume equivalent (copies L^{-1})			Average	Standard deviation
Replicate		**1**	**2**	3	1	2	3		
Jan 2023									
St. 1	2000	30.757	30.809	30.665	131,416	127,015	139,600	132,677.1	6,386.3
St. 2	2000	34.607	34.628	35.457	10,517	10,375	6,022	8,971.3	2,555.0
St. 3	2000	ND	ND	ND	0	0	0	0.0	0.0
St. 4	2000	ND	ND	ND	0	0	0	0.0	0.0
St. 5	2000	ND	ND	ND	0	0	0	0.0	0.0
St. 6	2000	ND	ND	ND	0	0	0	0.0	0.0
St. 7	2000	ND	ND	ND	0	0	0	0.0	0.0
St. 8	2000	35.836	ND	ND	4,696	0	0	1,565.4	2,711.3
Feb 2023									
St. 1	2000	34.116	34.961	34.886	14,510	8,335	8,758	10,534.2	3,449.3
St. 2	2000	36.464	36.399	ND	3,111	3,246	0	2,119.0	1,836.4
St. 3	2000	ND	ND	ND	0	0	0	0.0	0.0
St. 4	2000	ND	ND	ND	0	0	0	0.0	0.0
St. 5	2000	37.382	ND	ND	1,703	0	0	567.7	983.2
St. 6	2000	ND	ND	ND	0	0	0	0.0	0.0
St. 7	1000	ND	ND	ND	0	0	0	0.0	0.0
St. 8	2000	35.039	34.237	33.673	7,920	13,408	19,402	13,576.6	5,742.7
Mar 2023									
St. 1	2000	35.469	34.946	36.150	5,976	8,420	3,822	6,072.7	2,300.9
St. 2	2000	ND	ND	ND	0	0	0	0.0	0.0
St. 3	2000	ND	ND	ND	0	0	0	0.0	0.0
St. 4	2000	ND	ND	ND	0	0	0	0.0	0.0
St. 5	2000	ND	ND	ND	0	0	0	0.0	0.0
St. 6	2000	ND	ND	38.629	0	0	752	250.6	334.1
St. 7	1000	37.365	ND	ND	3,445	0	0	1,148.3	1,989.0
St. 8	2000	ND	ND	37.539	0	0	1,537	512.4	887.5
Negative control	1000	ND	ND	ND	0	0	0	0.0	0.0

[1]ND: not detected.

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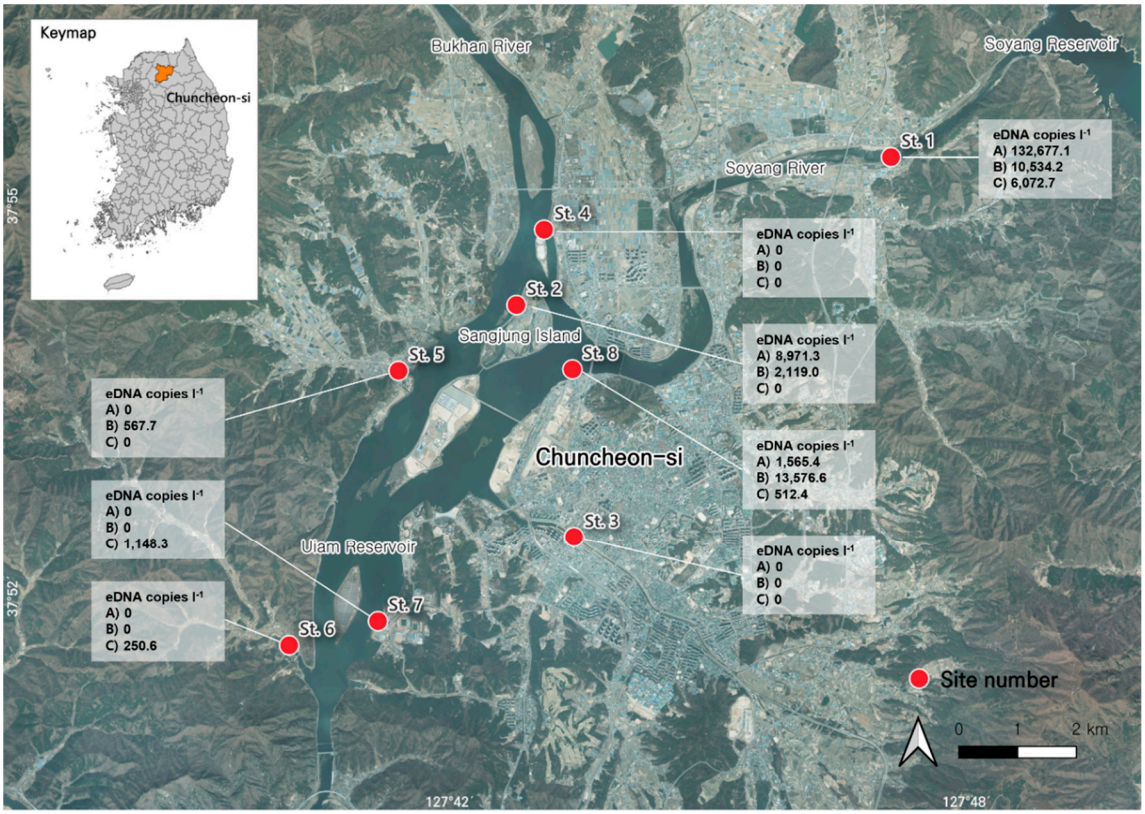

Figure 3. Average environmental DNA (eDNA) concentrations (copies L^{-1}) of the brown trout, *Salmo trutta*, quantified using the real-time PCR assay across eight stations in the downstream river of the Soyang Reservoir and around Uiam Reservoir. eDNA concentrations are represented by a heat map. A) January 2023, B) February 2023, and C) March 2023. Bathymetry is indicated by underlying isobars and shading.

Discussion

As the habitat range of *S. trutta*, a major invasive species in South Korea, expands, concerns are growing over the potential ecological disruptions in the Uiam Reservoir connected to the Soyang River, including habitat and food competition with native freshwater fish. Therefore, year-round spatio-temporal monitoring and habitat usage studies of this invasive species are necessary for effective management practices.

eDNA-based qPCR assays enhance management efforts aimed at preventing the establishment and spread of invasive species, and by providing rapid and precise results, is useful in protecting native biodiversity (Lodge et al. 2012; Thomsen and Willerslev 2015). The effectiveness of qPCR for species detection depends on the development of species-specific primers that exclusively amplify the DNA of the target species, minimizing false positives from cross-amplification with closely related species (Ficetola et al. 2008; Wilcox et al. 2013). To ensure target specificity, the primers need to be tested against all related species potentially present in the study area to confirm that only the target species is amplified. This validation process is essential for the accurate detection of species using molecular monitoring. Using the forward and reverse primers and hydrolysis probe specific to *S. trutta* that were modified from Carim et al. (2016), we aimed to amplify *S. trutta* eDNA from environmental water samples by qPCR assay, even if present at low concentrations, to allow for accurate and early detection of the target species.

It has been previously reported that qPCR targeting *S. trutta* is an important tool for detecting and monitoring this invasive species (Banks et al. 2016; Carim et al. 2016; Deutschmann et al. 2019; Hernandez et al. 2020). This method aids in tracking the spread of *S. trutta* and supports efforts to control its population and mitigate ecological damage. In this study, we modified the primer set (reducing the Tm of the forward and reverse primers) and hydrolysis probe (increasing the Tm of the

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hydrolysis) reported by Carim et al. (2016) to enhance qPCR sensitivity and specificity. Reducing the Tm of both primers improved their binding efficiency to eDNA at low temperatures, increasing the amplification efficiency, especially in low-concentration samples, and resulting in greater detection sensitivity. Additionally, increasing the Tm of the hydrolysis probe enhances its binding stability to the target sequence, ensuring more selective binding to the correct target and reducing nonspecific binding. This minimizes false positives and enables more accurate detection of the target species. Aligning the Tm of the primers and probe improved the overall balance and efficiency of the qPCR, thus enhancing the robustness of the qPCR assay, particularly when complex or degraded samples are used. These optimizations led to more reliable and precise detection of species, such as *S. trutta*, via molecular monitoring.

In the present study, *S. trutta* eDNA was detected in high quantities and frequencies in the upstream (St. 1) and downstream (St. 8) sections of the Soyang River, which is consistent with previous findings (NIE 2020, 2023; Park et al. 2022; Kim et al. 2023). These areas are characterized by shallow water depths, with riverbeds mainly composed of sand, pebbles, gravel, and boulders (Park et al. 2022; NIE 2023). In addition to these two stations, *S. trutta* eDNA was detected on Sangjung Island (St. 2), located upstream of the Uiam Reservoir. The environmental water was released from the middle layer of the Soyang Reservoir for hydroelectric power generation, maintaining a discharge water temperature of 15 °C throughout the year. As a result, the downstream area, approximately 10 km before merging with the Bukhan River, is influenced by the cold water released from the reservoir (Yi et al. 2006). These three stations were directly affected by cold water. Thus, the riverbed structure and hydraulic characteristics of the Soyang River provide an optimal environment for *S. trutta* inhabitation and spawning (Kondolf and Wolman 1993; Young 1995; Armstrong et al. 2003), because this species requires low water temperatures and high levels of dissolved oxygen. Park et al. (2022) observed sexually mature males and females in the Soyang Reservoir, supporting the conclusion that *S. trutta* migrates downstream from the Soyang Reservoir for spawning. Therefore, these areas serve as overwintering habitats for anadromous fish, such as *S. trutta*, which migrate to the upper rivers or streams for reproduction. This species also has a high potential to expand its habitat to the Paro Reservoir, located upstream of the Uiam Reservoir, because of its distinct life cycle, necessitating broader monitoring efforts across the Han River basin.

Our study confirmed previously known habitats of *S. trutta* and demonstrated that this fish is expanding its habitat. Consistent with previous findings, we found that *S. trutta* eDNA was detected in the Soyang River (Park et al. 2022; Kim et al. 2023); however, we also detected *S. trutta* in the Uiam Reservoir, indicating that the species utilizes a broader range of habitats than previously reported. Although the quantity of eDNAs does not always directly reflect the biomass or number of individuals, owing to various biotic and abiotic factors in the natural habitats of the target species (Goldberg et al. 2015; Barnes and Turner 2016; Deutschmann et al. 2019; Knudsen et al. 2019; Yates et al. 2019), our results highlight qPCR as an alternative tool for determining the presence of *S. trutta* in aquatic environments. This method offers a more efficient and effective approach for assessing and monitoring practices than traditional field surveys. Furthermore, this approach can support conservation efforts by habitats that require removal of invasive species with the goal of restoring the area to its predefined historical conditions (Banks et al. 2016).

This survey was conducted during a limited period, specifically during the winter months (January and February) and early spring (March), when water was at the lowest annual temperature (Park et al. 2022). These water temperatures are suitable for *S. trutta*, enabling broad species distribution. It is necessary to apply molecular monitoring during warmer months, that is, from late spring to fall, when water temperatures rise. During the period, it is likely that *S. trutta* move to deeper and cooler areas at the bottom of the Uiam Reservoir, where lower water temperatures are maintained. In this study, we found that *S. trutta* eDNA was more frequently detected at the southern stations (Stns. 5, 6, and 7), which were located around the main water body of the Uiam Reservoir in February and March, when the water temperature was higher than that in January.

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In addition, *S. trutta* frequently hybridizes with other native salmonid species (Verspoor 1988; Meldgaard et al. 2007; Castillo et al. 2008). It also has a high potential to hybridize with native salmonid species in South Korea, such as *B. lenok tsinlingensis* and *O. masou masou*, leading to genetic mixing that reduces the integrity and fitness of native populations. Such hybridization may result in the loss of locally adapted traits, inhibiting resilience to environmental changes, climate stress, and competition with invasive salmonid species. The introduction of *S. trutta* through intentional stocking or natural migration to other South Korean rivers and streams could negatively impact the natural genetic pollution, further endangering native populations. *S. trutta* fishing has gained popularity and attempts have been made to transplant this species into other rivers or streams in online communities, highlighting the need for ongoing monitoring to prevent its spread. Overall, our findings highlight the potential for the future use of our qPCR assay for the monitoring of invasive fish species.

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