



Article

# Development of a High-Sensitive qPCR-Based Molecular Diagnosis Method for Detection of *Clonorchis sinensis* in Fish Muscle and Environmental Water

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## Abstract

A liver fluke, *Clonorchis sinensis* is a representative fish-borne parasite infecting humans, and sensitive detection in fish hosts or aquatic environments is important for monitoring infection sources in endemic areas. Conventional diagnostic methods based on microscopy or conventional PCR often show limited sensitivity, particularly under low-parasite conditions. In this study, we developed a high-sensitive and species-specific molecular marker and established a real-time PCR (qPCR)-based diagnostic method targeting metacercariae isolated from freshwater fish, representing the transmission stage of *C. sinensis*. Primers and a hydrolysis probe targeting the mitochondrially encoded *cytochrome c oxidase 1* (*COI*) gene were designed, and all primer combinations produced stable amplifications with single melt curves in *C. sinensis*-positive samples. Among them, one combination was finally selected as the optimal marker due to its high specificity, including validation against mixed trematode samples to confirm species-specific detection. The qPCR assay showed excellent linearity ( $R^2 = 0.998$ ), with a detection limit of  $10^1$  copies per reaction and a quantification limit of  $10^2$  copies per reaction. In addition, the assay successfully detected *C. sinensis* DNA in environmental water samples spiked with metacercariae, demonstrating its applicability to aquatic samples for environmental surveillance purposes. Compared with conventional PCR, the developed qPCR method in this study exhibited markedly improved sensitivity in fish-derived samples. Overall, this qPCR assay provides a robust diagnostic tool for laboratory analysis and has potential utility for environmental DNA-based monitoring of clonorchiasis risk areas within a One Health framework.

**Keywords:** *Clonorchis sinensis*; real-time PCR (qPCR); metacercariae; molecular diagnosis; environmental DNA (eDNA)

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## 1. Introduction

A liver fluke, *Clonorchis sinensis*, known to have the highest rate of human infection, is a foodborne parasite commonly found in East Asia, including Korea and China. Human infection occurs through the consumption of raw freshwater fish infected with metacercariae [1]. *C. sinensis* is recognized as a parasite of public health importance, as chronic

infection can cause cholangitis, cholelithiasis, liver fibrosis, and biliary tract cancer [2]. In South Korea, persistent infections have been reported, particularly in river basins, making the management and prevention of *C. sinensis* infection a critical public health issue [3].

Although the definitive host of *C. sinensis* is mammals, including humans, they have a complex life cycle that involves passing through freshwater shellfish as the first intermediate host and freshwater fish as the second intermediate one to become adults. Eggs released in the feces of the definitive host are ingested by freshwater shellfish, where they develop into cercariae. Afterwards, they invade the muscles of freshwater fish and exist in the form of metacercariae enclosed in cysts [4]. Freshwater fish known as intermediate hosts of *C. sinensis* in South Korea include stonefish (*Pungtungia herzi*), Korean slender gudgeon (*Squalidus japonicus coreanus*), small gudgeon (*Microphysogobio yaluensis*), stone moroko (*Pseudorasbora parva*), and slender bitterling (*Acheilognathus lanceolatus*) [5].

The Korea Disease Control and Prevention Agency (KDCA) has been continuously promoting diagnostic testing and treatment distribution programs for residents in areas with high rates of *C. sinensis* infection. Simultaneously, the agency is conducting a survey of freshwater fish to assess the risk of foodborne parasite infection. An analysis of intestinal parasite infections in South Korea revealed that it had a higher infection rate than other parasites, with particularly high prevalence rates observed in the Seomjin and Nakdong River basins, suggesting that freshwater fish in these areas are key vectors [5].

The standard diagnostic method for *C. sinensis* infections is microscopic observation of eggs in stool samples. However, this method is time-consuming and labor-intensive, has low species-specificity, and has limited sensitivity in individuals with low infection rates. PCR-based diagnostic methods and serological tests have also been reported, but these have not been established as alternative diagnostic methods due to limitations such as sensitivity instability, difficulty distinguishing between past infections, and reliance on expensive equipment [3,6–8]. In particular, conventional PCR (cPCR) assays rely on agarose gel electrophoresis for final detection, which may limit sensitivity under low-template conditions and increase the risk of amplicon contamination under low-concentration template conditions. To overcome these limitations, this study developed a hydrolysis probe-based real-time PCR (qPCR) assay targeting the mitochondrially encoded cytochrome *c* oxidase 1 (COI) gene, which improves sensitivity, reduces contamination risk, and enables reliable detection even under low-concentration conditions.

Meanwhile, the primary method for investigating *C. sinensis* infection in fish involves artificial digestion of fish muscle and direct microscopic observation of metacercariae. While highly accurate, this method requires significant time and labor to collect and analyze large numbers of fish samples from natural environments, and detection efficiency is significantly reduced when infection intensity is low. Therefore, a new diagnostic approach is needed to overcome the labor-intensive nature and sensitivity limitations of existing fish diagnostic methods [3].

Recently, environmental DNA (eDNA), a technique that directly detects DNA from environmental samples, has been recognized as an effective tool for biodiversity monitoring and pathogen surveillance. eDNA can determine the presence of organisms in the environment without directly collecting them, and its potential application to parasite distribution surveys has been reported [9,10]. This approach could offer a novel strategy for proactively detecting the risk of liver fluke infections across natural aquatic systems [11,12].

In this study, we developed a qPCR-based diagnostic method capable of specifically detecting *C. sinensis* metacercariae in fish muscle and evaluated its sensitivity and efficacy. Furthermore, by applying eDNA techniques to explore the potential for detecting *C. sinensis* in environmental waters, we hope to contribute to the establishment of a future *C. sinensis* infection monitoring and disease management system.

## 2. Results

### 2.1. Evaluation of qPCR Amplification Performance and Selection of the Optimal Primer–Hydrolysis Probe Combination

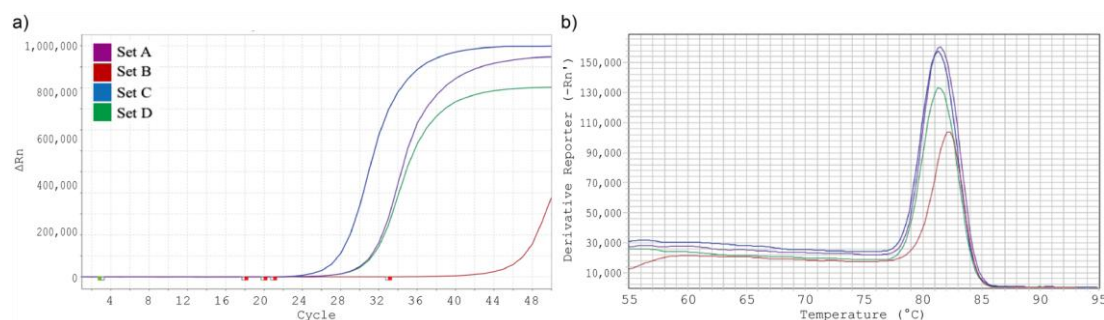
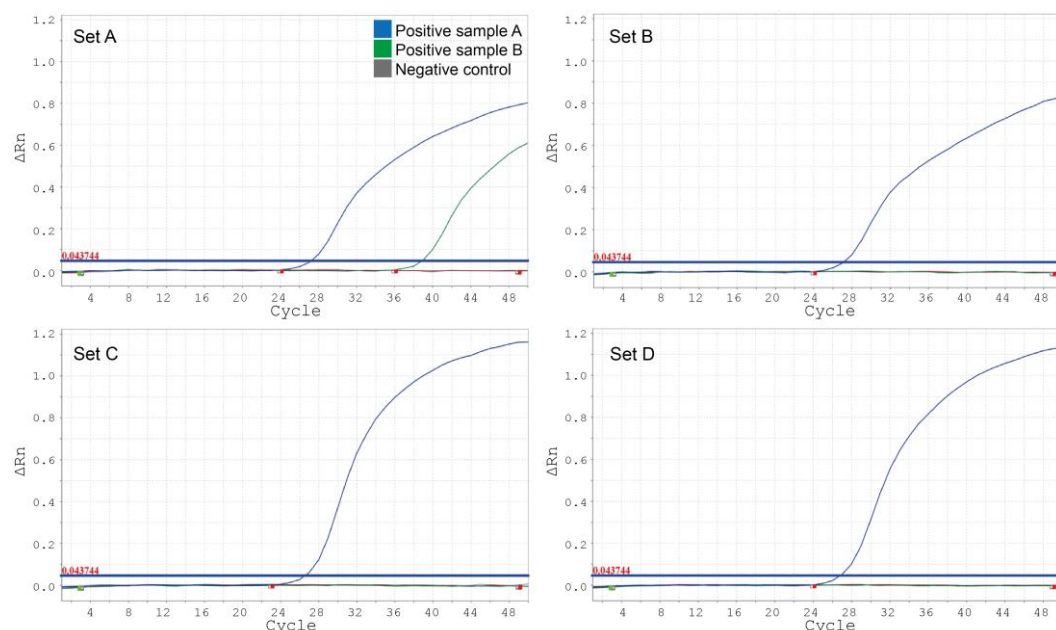
In this study, to develop an effective qPCR molecular marker for the detection of *C. sinensis*, species-specific primers and a hydrolysis probe were designed based on the DNA sequence matrix of the COI gene of mitochondrial genome sequences belonging to the class Trematoda retrieved from GenBank database in National Center for Biotechnology Information (Figure 1; Table 1). The mitochondrial genome sequence of *C. sinensis* (GenBank accession number KJ204588) was used as the reference sequence. To evaluate the amplification characteristics of the designed primers, a total of four primer pair combinations were constructed, and SYBR Green-based qPCR amplification reactions were performed using genomic DNA (gDNA) extracted from *C. sinensis* as a template. Though a relatively low amplification reactivity was observed in one primer combination (set B), a single peak was confirmed in the melt curve analysis for all primer combinations, confirming the formation of a *C. sinensis*-specific amplification product without nonspecific amplification (Figure 2). Accordingly, all designed primer combinations were judged to have basic species-specificity as qPCR primers for *C. sinensis* detection. To select one optimal marker, including the combination of primers and a hydrolysis probe, qPCR amplification reactivity were tested on the positive samples A and B containing *C. sinensis* individuals using all designed primer–hydrolysis probe combinations. As a result, qPCR amplification reactions were confirmed for all combinations for positive sample A. However, except for positive sample B containing a mixture of *C. sinensis* and other trematodes, specific amplification reaction was observed only with the primer–hydrolysis probe set A (Csi-COI-0384f, Csi-COI-0522r, and Csi-COI-0503p) (Figure 3). Based on these results, a set of primer–hydrolysis probe combinations that showed high specificity for *C. sinensis* even under mixed sample conditions was selected as the final molecular marker for the qPCR detection.

|                                               | Forward primer             | Hydrolysis probe  | Reverse primer            |
|-----------------------------------------------|----------------------------|-------------------|---------------------------|
| <i>Clonorchis sinensis</i> NC 012147          | TCTTCTGCTAGAGATTATAGAGGT   | CGGATAGCGGGTGAGG  | ACGACACTCT ATAATAGTTTGGGC |
| <i>Metorchis bilis</i> NC 079698              | C..G...T.C..G....C..G..A   | T....CA.AT..CCT.  | G..T....G .....TA.....    |
| <i>Metorchis orientalis</i> NC 028008         | G..A...T.GG.T.....G..G     | T.....C...A.TTT.  | G..T..T.A ..T....G.....   |
| <i>Metorchis xanthosomus</i> NC 079699        | ..A...T..G.T.....A         | T.....AT..TTT.    | G..T..T.A .....TA.C.....  |
| <i>Opisthorchis felinus</i> NC 011127         | C..G...T..G.T.....G..G     | T.....A.....TTT.  | G..T..T... ..C...A.....   |
| <i>Cryptocotyle lingua</i> NC 063968          | G....T.AG.T.....T..        | T.AG.GAT.ATACT.CT | ..TTTT..A .....T.C.....   |
| <i>Metagonimus yokogawai</i> NC 023249        | ..G..GT.AG.T.....G         | T..GG.AA.A.T...T  | ..CT.T..G .....C.....     |
| <i>Haplorchis taichui</i> NC 022433           | C..G...T...G.....G..       | T.AG..ATGATACC... | G..GT..... ..TG.G.....    |
| <i>Fasciola gigantica</i> NC 024025           | .....AG.TTG.....TCT..      | T.TGG.CGA..GT.CT  | T..T..TAG. ..TT.....      |
| <i>Paragonimus heterotremus</i> NC 039430     | .....G.G.G.T.....TCT..     | T.TG.GA..ATACTGT. | T..GAGG... ..T.....       |
| <i>Clinostomum complanatum</i> NC 027082      | CT.A...G.TTT.G....TCG..G   | T....CA.T..T.T..A | G..TATG..G G.G.....       |
| <i>Schistosoma japonicum</i> NC 002544        | .T.G...T..TT..C.AC.TCT..   | -----..TC.A...    | .T.TTCT..A G.T..TAGA..AT. |
| <i>Schistosoma mekongi</i> NC 002529          | GT.G...T..CA..C.ACGTCT..   | -----..TT.A.AT    | TT.TTCT..A G.T...AG...AT. |
| <i>Trichobilharzia regenti</i> NC 009680      | .T.A...AT.CAGAG..CG.TG..G  | -----AATC.T..T    | T..TGTT..G G.T.....T.     |
| <i>Trichobilharzia szidati</i> NC 036411      | GT.A...ATACA.A.A.CG.TG..G  | -----AATC.T..T    | T..GGTT..G G.TG.T.....    |
| <i>Schistosoma haematobium</i> NC 008074      | AT.A..CATATCT..GA..TC...   | -----..T..CGAT    | TAAGACT..G .....A.A...T.  |
| <i>Schistosoma indicum</i> NC 047240          | .T.G...AAT..TG..GAG.T.T..G | -----..T..A.AA    | TAAGACT..G ..T..A...T.    |
| <i>Schistosoma mansoni</i> NC 002545          | .T.A...AAT.T...GGGTCT..    | -----..TT.A..A    | CAA.TGT..G .....A.....    |
| <i>Schistosoma spindale</i> NC 008067         | .T.A...ATA.T...GGG.TCT..G  | -----..TA.T.AA    | GA.TTCT..G .....A.....    |
| <i>Posthodiplostomum commutatum</i> NC 044643 | AT.A...T..GTT.G.....G..G   | T.CGAGAT.CTT.AGT. | G..GTTG... G.....G...G    |
| <i>Cyathocotyle prussica</i> NC 039780        | .T.A...AT.CG.G.GG..C.CG... | T...A.ATTAA.A.TT  | ..TTTA..A .....G.TA...A.. |
| <i>Diplostomum ardeae</i> NC 049068           | .T.G...AG.TTTCC.....C..G   | TTTT.TATTTA.T.AT  | T...GTT... ..T..T.....    |
| <i>Apharyngostreia pipientis</i> NC 059570    | GT.A...AG.GTTCCG...GCC...  | TTTT.TCT..TT..T.T | T..TATT... ..T.....       |
| <i>Azygia hwangsiyui</i> NC 049873            | CT.G...T.GGTG.GA...CTCT..  | GT.GGCCTTAT.CTGTT | ..G..T..G ..T..T..G.....  |
| <i>Azygia robusta</i> NC 082541               | C....AT.CG...C...TCT..G    | G..G..C..AT..TGTT | ...T..T... ..TT.G.....    |

**Figure 1.** Alignment matrix of forward and reverse primers and hydrolysis probe specific to *Clonorchis sinensis* from the mitochondrially encoded cytochrome c oxidase 1 (COI) gene region. Oligonucleotide sequence of the reverse primer should be reverse complement from the binding site. Dots represent the identical oligonucleotide bases of *C. sinensis* with the other species belonging to the class Trematoda, including all reference sequences of mitochondrial genomes of the order Opisthorchiida and each two representative sequences of the other orders (Azygiida, Diplostomid, Plagiorchiida, and Strigeidida).

**Table 1.** Design and characteristics of candidate real-time PCR primers and hydrolysis probe targeting the mitochondrially encoded cytochrome *c* oxidase 1 (COI) gene of *Clonorchis sinensis*.

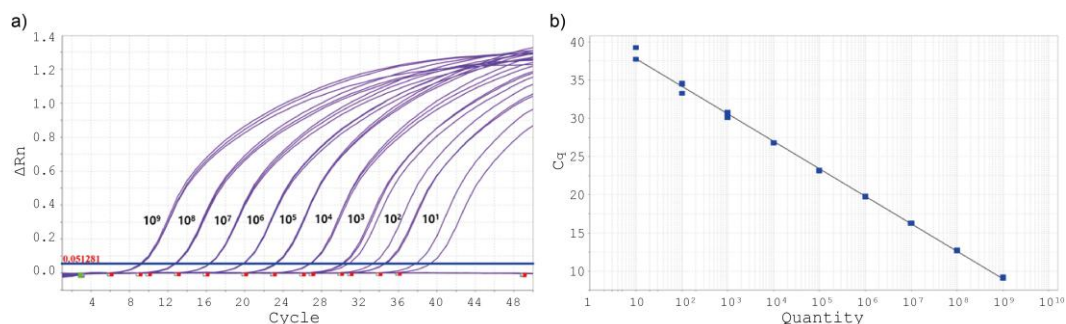
| Primer/<br>Hydrolysis Probe | Type    | Sequence<br>(5' → 3')      | Length<br>(bp) | GC (%) | T <sub>m</sub> (°C) | Target<br>Position |
|-----------------------------|---------|----------------------------|----------------|--------|---------------------|--------------------|
| Csi-COI-0384f               | forward | TCTTTCTGCTAGAGATTATAGAGGT  | 25             | 36     | 57.16               | 384–408            |
| Csi-COI-0437f               | forward | TTCATTGGCTGGGTATCG         | 20             | 45     | 56.98               | 437–456            |
| Csi-COI-0522r               | reverse | GCCCAAATATTATAGAGTGTCTGT   | 24             | 42     | 59.00               | 522–545            |
| Csi-COI-0564r               | reverse | CAACGTACCCAACAACAAG        | 19             | 47     | 55.20               | 564–582            |
| Csi-COI-0503p               | probe   | FAM-CGGATAGGCGGGTGAGG-BHQ1 | 17             | 71     | 60.04               | 503–519            |

**Figure 2.** SYBR Green-based real-time PCR amplification (a) and melting curve (b) analysis of newly designed PCR primer sets evaluated using *Clonorchis sinensis* positive sample A.**Figure 3.** Real-time PCR amplification profiles obtained using four different PCR primer sets (A–D sets) combined with a probe for the detection of *Clonorchis sinensis* in positive reference samples.

## 2.2. Standard Curve and Limit of Detection (LOD)

To evaluate the quantitative performance and detection limit of the *C. sinensis*-specific qPCR molecular marker, a qPCR amplification reaction was performed using a DNA sequence of the COI region as a positive control sample prepared through gene synthesis, and a standard curve was generated. As a result, the coefficient of determination ( $R^2$ ) of the calibration curve created through linear regression analysis between the  $C_q$  value and template copy number was 0.998, confirming that the *C. sinensis*-specific molecular marker in this study exhibited quantitative performance with excellent linearity and reliability. In addition, the qPCR amplification signal was stably detected up to  $10^1$  copies per reaction

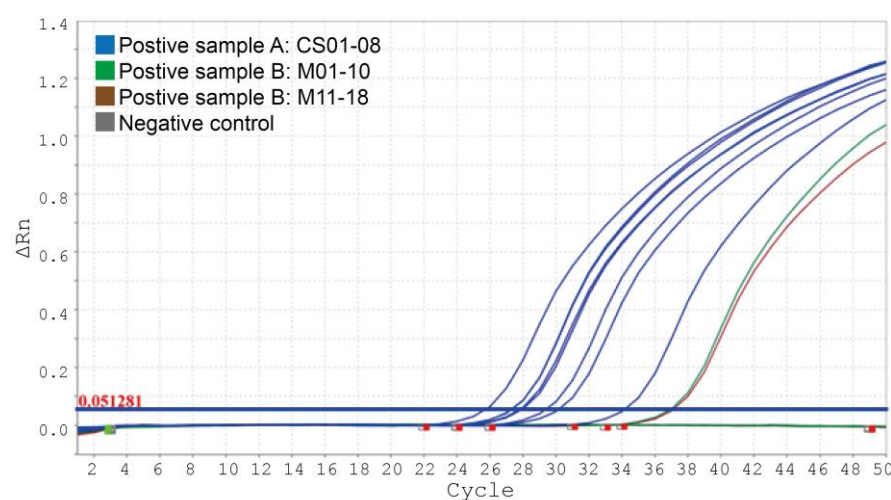
(copies/rxn), and the LOD was confirmed to be  $10^1$  copies/rxn, and the minimum limit of quantification (LOQ), which is the minimum concentration at which quantitation is ensured, was confirmed to be  $10^2$  copies/rxn (Figure 4).



**Figure 4.** Real-time PCR amplification profiles and standard curve for the *Clonorchis sinensis*-specific molecular marker. (a) qPCR amplification plots generated using 10-fold serial dilutions of a synthetic positive control targeting the mitochondrially encoded *cytochrome c oxidase 1* (COI) gene of *C. sinensis*. Amplification curves show a concentration-dependent shift in  $C_q$  values, while no amplification was observed in the  $10^0$  and  $10^{-1}$  copy/rxn. (b) Standard curve constructed by plotting  $C_q$  values against the logarithm of template copy numbers. The standard curve exhibited excellent linearity ( $R^2 = 0.998$ ), demonstrating reliable quantitative performance of the assay. The limit of detection (LOD) was determined to be  $10^1$  copies/rxn, and the limit of quantification (LOQ) was  $10^2$  copies/rxn.

### 2.3. Specificity of the qPCR Marker

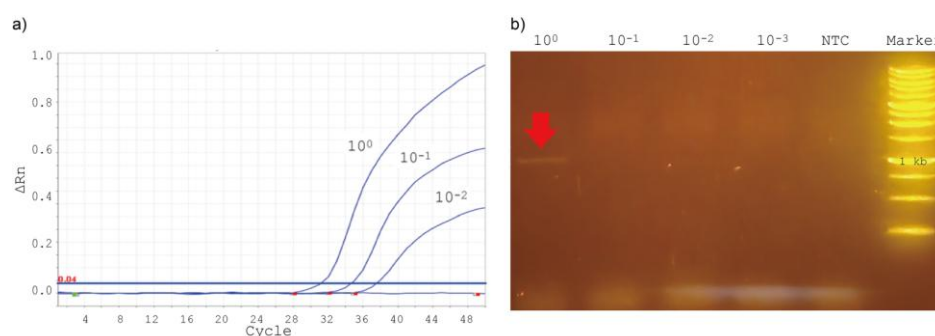
The qPCR amplification reaction was performed on each individual from the positive sample A morphologically classified as *C. sinensis* metacercariae and the positive sample B containing a mixture of *C. sinensis* metacercariae and other metacercariae. As a result, all eight individuals (CS-01 to CS-08) isolated from positive sample A were confirmed to be qPCR-positive. Among the 18 individuals isolated from positive sample B, two samples showed qPCR positive reactions, and the remaining samples did not show qPCR amplification reactions, so they were determined to be other trematod species rather than *C. sinensis* (Figure 5). These results demonstrate that the qPCR marker developed in this study has high specificity for *C. sinensis* and is generally consistent with the morphological classification results.



**Figure 5.** Real-time PCR amplification plots for specificity evaluation of the *Clonorchis sinensis*-specific marker. Amplification was observed in *C. sinensis* samples, whereas no amplification was detected in non-*C. sinensis* trematodes or the no-template control (NTC).

#### 2.4. Sensitivity of the qPCR Marker

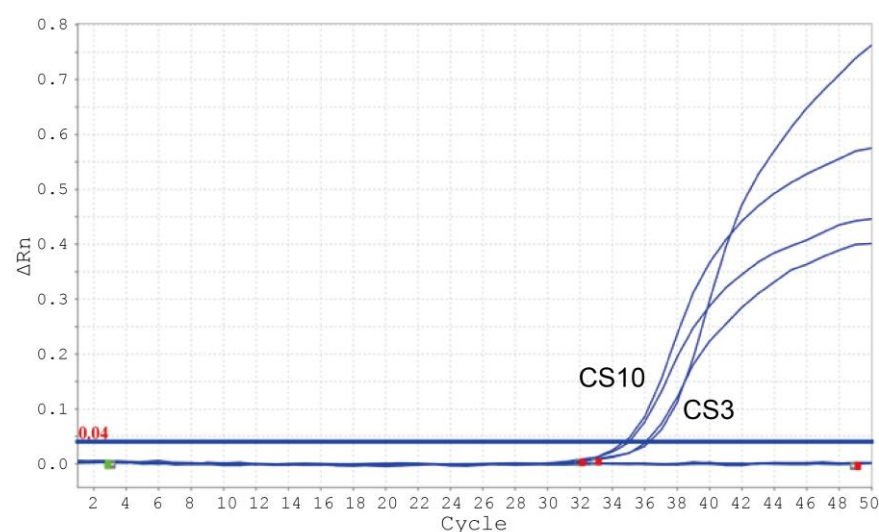
A sensitivity comparison experiment between qPCR and cPCR was performed using gDNA extracted from *C. sinensis* metacercariae. qPCR analysis used 10- and 100-fold serially diluted samples of the original solution (3 ng/ $\mu$ L) of gDNA extracted from *C. sinensis*. Positive amplification reactions were observed in both the original solution and the 10- and 100-fold diluted samples. However, no qPCR amplification reaction was observed in the 1000-fold diluted sample. In contrast, cPCR analysis revealed a positive amplification band only in the original DNA solution among all samples, while no amplification reaction was observed in the diluted samples. Taken together, these results demonstrate that the *C. sinensis*-specific qPCR marker in this study possesses a detection sensitivity at least 100-fold higher than that of the existing cPCR method (Figure 6).



**Figure 6.** Sensitivity comparison between real-time PCR (a) and conventional PCR (b) for detection of *Clonorchis sinensis* using serially diluted genomic DNA. The red arrow indicates the positive band in the undiluted DNA sample (10<sup>0</sup>).

#### 2.5. Feasibility of Environmental DNA-Based Detection Using the qPCR Marker

To evaluate the applicability of the qPCR marker developed in this study, qPCR amplification reactions were performed by adding various concentrations of *C. sinensis* metacercariae to 1 L of environmental water. As a result, positive qPCR amplifications were confirmed in environmental water samples (CS3, CS10) containing 3 and 10 metacercariae, respectively. In contrast, no qPCR amplification was observed in the sample without *C. sinensis* metacercariae (CS0) and the environmental water samples containing 1 or 5 metacercariae (CS1, CS5) (Figure 7).



**Figure 7.** Real-time PCR amplification plots of environmental water samples spiked with *Clonorchis sinensis* metacercariae. Positive amplification was detected in samples

containing 3 (CS3) and 10 (CS10) metacercariae, while no amplification was observed in the negative control or samples containing 1 or 5 metacercariae.

### 3. Discussion

In this study, we developed a qPCR-based molecular marker capable of specifically detecting *C. sinensis* with high sensitivity. This marker holds promise as a molecular diagnostic tool that can improve nonspecific reactions and low detection sensitivity, issues previously identified in morphological observations and cPCR-based diagnostics.

All four primer pairs designed in this study exhibited qPCR amplification in *C. sinensis*-positive samples, and melt curve analysis confirmed the formation of a single amplification product. These results suggest that all designed primer combinations can be used as molecular markers for *C. sinensis*-specific qPCR. Evaluation of the amplification characteristics of primers and hydrolysis probe combinations revealed that set A (Csi-COI-0384f, Csi-COI-0522r, and Csi-COI-0503p) showed stable amplification reactions not only in positive sample A consisting of a single species of *C. sinensis*, but also in positive sample B containing a mixture of *C. sinensis* and other trematode metacercariae. In contrast, the remaining primer combinations showed positive reactions in all positive sample A, but no amplification reactions were observed in positive sample B. These results indicate that primer–hydrolysis probe combination set A can selectively detect *C. sinensis* though the concentration of *C. sinensis* in the DNA extracted from the mixed sample is low. Based on this, set A was finally selected as the optimal molecular marker combination for qPCR detection of the parasite in this study.

The quantitative performance of the selected qPCR markers was systematically evaluated, and the LOD and LOQ were determined. This qPCR assay demonstrated excellent linearity over a wide quantitative range, and the  $R^2$  was 0.998, confirming excellent reliability and reproducibility of quantitative analysis. The LOD was  $10^1$  copies/rxn, and the LOQ was  $10^2$  copies/rxn, demonstrating high analytical sensitivity.

Furthermore, we compared the detection sensitivity with cPCR-based diagnostic methods, which are currently utilized for *C. sinensis* monitoring. While cPCR-based diagnostic methods can result in false negative results at low template concentrations, the qPCR marker developed in this study demonstrated stable detection even under low copy number conditions, significantly improving diagnostic sensitivity. This characteristic is expected to be particularly useful in conditions where detection is difficult with conventional diagnostic methods, such as low-intensity infections with low parasite counts or early stages of infection.

Additionally, the low LOD identified in this study supports the applicability of eDNA-based monitoring. Because parasite-derived DNA is likely to be present in trace amounts in environmental water samples, this qPCR assay, which can reliably detect up to  $10^1$  copies/rxn, can be an effective tool for noninvasively monitoring *C. sinensis* contamination in aquatic environments. Overall, our findings suggest that the qPCR molecular marker developed in this study is a useful tool suitable not only for laboratory diagnostics but also for quantitative analysis in epidemiological investigations and environmental monitoring.

Previous molecular diagnostic studies on *C. sinensis* have been conducted mainly in China, targeting human fecal samples, and qPCR techniques targeting COI gene or nuclear 18S or 28S ribosomal RNA gene regions have been reported to have higher sensitivity than microscopic egg detection methods [13]. Although these molecular diagnostic methods are useful for assessing low-infectivity or residual infection after treatment, most of them focus on human diagnosis of adult parasite infection and thus have structural limitations in tracing the source of infection or assessing transmission patterns in the environment [11–14]. Unlike conventional human-centered diagnostic strategies, this study

is distinguished by the application of a qPCR-based detection approach targeting metacercariae isolated from freshwater fish, which serve as the second intermediate host in the life cycle of *C. sinensis*. Despite being a direct cause of human infection, *C. sinensis* metacercariae has traditionally been identified primarily through morphological observation. This process, however, requires skilled personnel and considerable time [15]. The molecular diagnostic method developed in this study enables objective and sensitive detection even in cases where morphological identification is difficult. Notably, all morphologically confirmed *C. sinensis* metacercariae (8/8) were accurately detected by this method, corresponding to a diagnostic sensitivity of 100% under experimental conditions. Collectively, these findings highlight the novelty of this study, which (1) targets metacercariae stages in fish hosts instead of focusing solely on adult human parasites, (2) validates species-specific detection using mixed liver fluke samples, and (3) extends molecular diagnostics to environmental monitoring through water quality analysis.

Furthermore, the qPCR technique established in this study demonstrates its applicability to environmental water samples, expanding the diagnostic scope of *C. sinensis* detection to the environmental level. Recently, research utilizing eDNA for aquatic parasite and pathogen surveillance has been increasing, offering the advantage of noninvasively predicting potential outbreak areas [11,16]. In particular, an eDNA-based qPCR study targeting schistosomiasis has demonstrated that endemic areas can be monitored using only environmental water samples [17], and this approach is considered highly applicable to *C. sinensis* surveillance. However, several limitations must be considered when applying this qPCR assay to eDNA-based surveillance. First, eDNA can persist in water even after parasite death, which does not necessarily indicate active transmission. Second, eDNA copy number may not directly reflect infection intensity or parasite burden in natural environments. Furthermore, environmental inhibitors such as humic substances can affect amplification efficiency, and eDNA movement in flowing water systems can complicate spatial interpretation of infection sources. Therefore, molecular detection results should be interpreted in conjunction with ecological and epidemiological data.

In summary, this study presents a novel approach that complements existing clonorchiasis management strategies by expanding the scope of clonorchiasis molecular diagnosis beyond human diagnosis to fish and aquatic environment monitoring. A molecular diagnostic system that simultaneously considers fish metacercariae and environmental waters can be developed into a “One Health” disease surveillance strategy that links human health, seafood safety, and aquatic ecosystem management. This approach is expected to contribute to the establishment of early warning systems and tailored quarantine strategies in endemic areas [2].

The qPCR-based molecular marker developed in this study showed high specificity and sensitivity for DNA derived from *C. sinensis* under laboratory conditions, but its application to field samples reflecting actual natural environments requires further validation. Notably, this study was conducted based on samples obtained under limited experimental conditions. Therefore, future field validation using this marker in environmental water samples collected from natural aquatic environments and various freshwater fish species is warranted. Such field-based studies will allow for a more definitive assessment of the practical applicability of this diagnostic technology and its effectiveness as a surveillance tool for epidemic areas.

## 4. Materials and Methods

### 4.1. Development of a *C. sinensis* Detection Molecular Marker

#### 4.1.1. Design of COI-Targeted Primers and Hydrolysis Probe for qPCR

Primer candidates for qPCR analysis were designed based on the DNA sequence of the COI region of *C. sinensis* (GenBank acc. no. KJ204588). The design of primers and a hydrolysis probe was comprehensively analyzed using the Sequence Manipulation Suite: PCR Primer Stats ([https://www.bioinformatics.org/sms2/pcr\\_primer\\_stats.html](https://www.bioinformatics.org/sms2/pcr_primer_stats.html) (accessed on 10 August 2024)) based on factors such as sequence length, GC content, melting temperature ( $T_m$ ), and possibility of secondary structure formation. PCR primers were designed to be 19–25 mer in length and 36–47% in GC content, and the  $T_m$  value was adjusted to be within 60–63 °C. The hydrolysis probe was designed to be 17 mer in length, limited to 71% GC content or less, and set to be 1–4 °C higher than the primer  $T_m$  value to improve binding specificity and amplification efficiency (Table 1).

#### 4.1.2. Verification of Primer Amplification Reactivity and Selection of Optimal Combination

To determine the amplification reactivity of the designed primer candidates and to verify the optimal combination, qPCR analysis was performed using *C. sinensis* positive samples. The positive samples were kindly provided by Professor SeongJun Choe of Chungbuk National University, South Korea. Positive sample A consists of metacercariae of a single species of *C. sinensis*, and positive sample B contains a mixture of *C. sinensis* and other trematod species (e.g., *Metagonimus yokogawai*, *M. takahashii*). gDNA was extracted from each sample, quantified at the same concentration, and used as a template for qPCR amplification.

First, SYBR Green-based qPCR was performed using four combinations of primer candidates designed to compare and analyze primer amplification characteristics (Csi-COI-0384f and Csi-COI-0522r; set A, Csi-COI-0384f and Csi-COI-0564r; set B, Csi-COI-0437f and Csi-COI-0522r; set C, Csi-COI-0437f and Csi-COI-0564r; set D). Each primer combination was subjected to qPCR on positive sample A, and the suitability of the primer combination was primarily verified by comprehensively evaluating the presence of non-specific amplification, amplification efficiency, and formation of a single amplification product by analyzing the amplification curve and melt curve. SYBR Green-based qPCR reactions were performed using Power SYBR® Green PCR Master Mix (Thermo Scientific, Waltham, MA, USA), and the total reaction volume (20 µL) contained 10 µL of 2× SYBR Green Master Mix, 1 µL each of forward and reverse primers, and 1 µL of template gDNA. The reaction conditions included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All qPCR reactions were performed using Applied Biosystems™ QuantStudio™ 5 (Thermo Fisher Scientific, USA).

Subsequently, qPCR analysis was conducted using the same four primers–hydrolysis probe combinations to determine the optimal combination probe-based qPCR reactions were performed using GoTaq® Probe qPCR MasterMix (Promega, Madison, WI, USA), and the total reaction volume of 20 µL contained 10 µL of 2× GoTaq Probe qPCR Master Mix, 1 µL of CXR reference dye, 1 µL of Template gDNA, 1 µL each of forward and reverse primers, 0.5 µL of hydrolysis probe, and 5.5 µL of Nuclease-free water. qPCR amplification reaction conditions were as follows: 1 cycle of initial denaturation at 95 °C for 2 min, 50 cycles of denaturation at 95 °C for 15 s, and ligation/elongation at 60 °C for 45 s, using Quantstudio™ 5 (Thermo Fisher Scientific). Through this process, the primer–hydrolysis probe combination with the best amplification efficiency and signal stability was selected as the final qPCR marker and used for subsequent analyses.

#### 4.2. Validation of the *C. sinensis* qPCR Marker

##### 4.2.1. Quantification and Limit of Detection (Standard Curve and LOD)

To evaluate the quantitative performance and LOD of the *C. sinensis*-specific molecular marker finally selected in this study, qPCR amplification reactions were performed, and a standard curve was generated. For this purpose, a DNA sequence of the *COI* region containing the oligonucleotide sequence of the species-specific molecular marker was generated through gene synthesis and used as a positive control. The synthesized positive control was adjusted to an initial concentration of  $10^9$  copies/ $\mu$ L and then diluted 10-fold to  $10^{-1}$  copies/ $\mu$ L to prepare a series of validation samples. The control material at each dilution step was used as a template to perform qPCR amplification reactions, and a calibration curve was created based on the obtained  $C_q$  values. Through this, the amplification efficiency, linearity, and detection limit of the species-specific qPCR marker was evaluated.

##### 4.2.2. Specificity of the *C. sinensis* qPCR Marker

To verify the specificity of the developed qPCR marker, qPCR analysis was performed on morphologically classified trematode individuals. The samples used for the specificity test were positive sample A (a single species of *C. sinensis*) and positive sample B (a mixture of metacercariae of various species including *C. sinensis*) used in Section 4.1.2. Eight individuals (CS-01 to CS-08) from positive sample A and 18 individuals (M-01 to M-18) from positive sample B were placed in 1.5 mL microtubes, one for each individual, and gDNA was extracted. gDNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted gDNA was stored at  $-20$  °C until analysis after measuring the concentration.

qPCR analysis to verify specificity was performed using the primer set A (Csi-COI-0384f and Cso-COI-0522r) and fluorescent probe (Csi-COI-0503p) finally selected as the *C. sinensis*-specific molecular marker. qPCR amplification reactions were performed using gDNA extracted from each individual as a template, and the specificity of the marker was evaluated based on whether a specific amplification signal appeared only in individuals morphologically classified as trematod species.

##### 4.2.3. Comparative Sensitivity Analysis Between qPCR and cPCR

To evaluate the sensitivity of the developed qPCR diagnostic marker, a sensitivity comparison experiment was conducted between the cPCR diagnostic marker used for *C. sinensis* detection and the newly developed qPCR diagnostic marker in this study. The gDNA used in the sensitivity comparison was extracted from two *C. sinensis* metacercariae using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The initial concentration of extracted gDNA was 3 ng/ $\mu$ L. The extracted gDNA was serially diluted 10-fold, 100-fold, and 1000-fold. 5  $\mu$ L of gDNA from each dilution was used as a template for cPCR and qPCR analyses, respectively. cPCR analysis was performed using primers RT5-1 (5'-ACTTCATCGAGTCATTGGTCGT-3') and RH3 (5'-CGTACTGTAAACGCGTTTGTGCA-3'), which target the retrotransposon gene of *C. sinensis* [18]. The expected size of the PCR amplification product using this primer combination was 929 bp, and the amplification results were confirmed by agarose gel electrophoresis. An analysis using the developed qPCR marker in this study was performed using gDNA from the same dilution level. The amplification sensitivity and detectable range at each dilution level were compared between cPCR and qPCR to assess whether the newly developed qPCR marker improved sensitivity.

#### 4.2.4. Assessment of eDNA Detection Using Spiked Water Samples

To demonstrate the detection of *C. sinensis* in environmental water, a spike test was performed in duplicate. Environmental water was spiked with 0 (CS0), 1 (CS1), 3 (CS3), 5 (CS5), and 10 (CS10) metacercariae, respectively, at each concentration. The samples were stored at  $-80^{\circ}\text{C}$  until use. All spike samples were filtered through glass-fiber filter paper ( $0.12\text{ }\mu\text{m}$ ), and eDNA was extracted from the filter. qPCR analysis was performed using the extracted eDNA. The reaction solution for qPCR analysis and qPCR amplification reaction conditions was the same as in Section 4.1.2.

## 5. Patents

A patent application related to the qPCR-based molecular diagnostic method described in this study has been filed in the Republic of Korea and is currently pending.

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**Conflicts of Interest:** Authors Jung Soo Heo and Keun-Yong Kim were employed by the company AquaGenTech Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Abbreviations

The following abbreviations are used in this manuscript:

|                    |                                                               |
|--------------------|---------------------------------------------------------------|
| <i>C. sinensis</i> | <i>Clonorchis sinensis</i>                                    |
| qPCR               | quantitative polymerase chain reaction                        |
| cPCR               | conventional polymerase chain reaction                        |
| COI                | mitochondrially encoded cytochrome <i>c</i> oxidase subunit 1 |
| eDNA               | environmental DNA                                             |
| $R^2$              | coefficient of determination                                  |
| LOD                | limit of detection                                            |
| LOQ                | limit of quantitation                                         |

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