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A simple and cost-effective extraction for piscine environmental DNA metabarcoding using guanidine hydrochloride method ☆,☆☆

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ABSTRACT

Environmental DNA (eDNA) metabarcoding is a valuable tool for assessing aquatic biodiversity, but the high cost and complexity of DNA extraction pose challenges for widespread adoption, especially in developing countries. This study presents a cost-effective eDNA extraction method using a guanidine hydrochloride (GuHCl) buffer, proteinase-K digestion, and isopropanol precipitation to improve the detection of fish communities. Comparison with the Qiagen DNeasy Blood & Tissue Kit using MiFish universal primers showed that the GuHCl protocol detected more fish species in freshwater samples, with comparable performance in relative read abundance metrics. However, the GuHCl method exhibited higher PCR inhibition in brackish samples, likely due to salinity and natural inhibitors. The results suggest that the GuHCl-based method is a viable alternative, offering enhanced sensitivity for low-abundance species in freshwater samples and cost savings. This protocol facilitates large-scale eDNA metabarcoding for ecological studies and conservation management efforts.

- The GuHCl protocol identified a greater diversity of fish species in freshwater samples than the Qiagen kit, but detected fewer species in brackish water samples.
- Both extraction methods demonstrated robust positive correlations in metrics of relative read abundance.

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Resource availability	The raw reads were available in GenBank under BioProject PRJNA1100780 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1100780).

Background

In the realm of aquatic ecology and biodiversity monitoring, the application of environmental DNA (eDNA) metabarcoding has emerged as a powerful tool for non-invasive species detection and assessment [1]. This cutting-edge technique relies on the extraction and analysis of genetic material shed into the environment by organisms, providing valuable insights into the diversity and abundance of aquatic species [2]. Piscine environmental DNA metabarcoding, in particular, has shown great promise for elucidating the intricate dynamics of fish communities within aquatic ecosystems [3,4], including evaluations pertaining to spawning activity [5,6], space and time variations [7], and habitat connectivity assessment [8].

Despite methodological considerations comprising nearly 50 % of research inquiries in fish metabarcoding, laboratory procedures such as eDNA concentration and extraction methods were only addressed in 12 % of these investigations [9]. Recent findings highlight the substantial influence of extraction protocols on eDNA detection, as demonstrated by previous studies [10,11]. The widespread adoption of eDNA metabarcoding faces a critical bottleneck, including the cost and efficiency associated with DNA extraction methods. It is noteworthy that DNA extraction constitutes one of the most substantial expenses in eDNA analysis, second only to sequencing costs [12]. The commercial kit methodologies often involve expensive reagents [13], hindering the scalability and accessibility of eDNA metabarcoding in resource-limited settings, such as implementation in developing countries. Moreover, the extraction method exerted the most significant influence on eDNA analysis, expending greater effect compared to variations in filter type [14]. Extraction of DNA from environmental samples presents unique challenges, as the success of eDNA extraction hinges not only on the quality and quantity of DNA obtained but also on achieving broad taxon coverage, which is crucial in metabarcoding studies. Thus, the extraction process is a critical factor determining the technique's overall efficacy. Notably, increased eDNA concentration does not always correlate with higher fish diversity in eDNA metabarcoding studies [15]. As the demand for large-scale environmental monitoring programs grows [16], driven by concerns over climate change and habitat degradation, scalable and economical eDNA extraction methods become imperative.

While most existing methodologies focused on species-specific studies [17], the number of eDNA extraction specifically for fish metabarcoding was limited. Previous non-commercial extraction using the phenol-chloroform method was compared to commercial extraction kit, revealing inferior detection of operational taxonomic unit (OTU) numbers and different community structures of vertebrates [18]. This study addresses this challenge by introducing a tested, cost-efficient eDNA extraction protocol tailored specifically for piscine eDNA metabarcoding. Our objective was to develop and rigorously test an extraction method that delivers results comparable to those obtained with commercial extraction kits, specifically using MiFish metabarcoding on water samples. Implementing a cost-effective extraction method for eDNA metabarcoding will facilitate the adoption of next-generation biomonitoring on a routine and large-scale basis. Additionally, we highlight potential limitations and considerations associated with the new eDNA extraction protocol.

Method details

Water collection and filtration

Water samples were obtained from two distinct locations along the Porong River: the upper freshwater segment (coordinates: 7°33′28.5″S 112°40′07.2″E) and the lower brackish estuarine segment (coordinates: 7°33′49.5″S 112°52′06.3″E) on April, 23th 2023. The decontamination and filtration blank protocol employed during water sampling was performed as documented in the previous study [1]. A composite sample comprising three liters of surface water collected from both the bank and central regions of each site was obtained using sterile equipment and thoroughly mixed to ensure homogeneity. Subsequently, the composite sample was rapidly stored in an ice until the filtration step. Six replicate filtrations were performed for each sample, wherein 500 mL aliquots were individually filtered through 0.45 μm GN-6 Metricel membranes (PALL Life Sciences, USA) using borosilicate vacuum filtration. Notably, each filtered membrane was specifically designated for subsequent DNA extraction procedures.

DNA extraction protocols

Genomic DNA was extracted employing two different extraction protocols, namely a non-commercial guanidine hydrochloride (GuHCl) protocol (henceforth referred to as the GuHCl protocol) and a commercial kit (Qiagen DNeasy Blood & Tissue). The triplicated membrane subsamples were used for the material of respective protocols. Extraction using the commercial kit was performed according to the manufacturer's manual and DNA was eluted in 50 μL of TE buffer. For the GuHCl eDNA extraction, a custom mod-

Table 1
Comparison in methods for extracting eDNA between the reference and this study procedures.

Step or procedure	reference procedures	This study
DNA source	Blood cells	eDNA on filter paper
Pre-lysis/ lysis buffer	10 mM NaCl, 10 mM EDTA	100 mM Tris, 100 mM EDTA, 10 mM NaCl, and 0.5 % sodium dodecyl sulphate
Filter treatment	None	shredded by FastPrep-24^{TM} Classic Instrument
DNA precipitation	Ethanol at room temperature	Chilled isopropanol
Air drying	No	Yes
DNA collection after precipitation	Spooling	Centrifugation at 13,000 rpm for five minutes at 4 ˚C

ification of the extraction protocol [19] was employed as follow and the comparison between reference and our modified GuHCl protocols are summarized in Table 1.

GuHCl protocol

1. Add 400 μL lysis buffer (100 mM Tris, 100 mM EDTA, 10 mM NaCl, and 0.5 % sodium dodecyl sulphate) to a 2 mL tube containing membrane
2. Add 2 pieces of 6.35 mm diameter ceramic spheres and shred using FastPrep-24^{TM} Classic Instrument (MP Biomedicals, Irvine, CA, USA).
3. Add 50 μL of proteinase-K and incubate at 60 ˚C for an hour.
4. Centrifuge at 13,000 rpm for five minutes.
5. Transfer the resulting supernatant to a new 1.5 mL tube and add with 400 μL of guanidine hydrochloride (GuHCl) buffer containing 4 M GuHCl and 0.5 M potassium acetate at pH 4.2, and incubate at 60 ˚C for ten minutes.
6. Centrifuge at 13,000 rpm for five minutes.
7. The supernatant was transferred to a new 1.5 mL tube and gently mix with 0.7 to 0.8 times its volume of chilled isopropanol.
8. Incubate the mixture at –20 ˚C for two hours.
9. Centrifuge at 13,000 rpm for five minutes at 4 ˚C for DNA pelleting.
10. Discard the supernatant with a pipette, and wash the DNA pellet with 1 mL of 70 % ethanol.
11. Centrifuge at 13,000 rpm for five minutes at 4 ˚C.
12. Discard the ethanol carefully with a pipette and remove the excess ethanol by air-drying for 10 min.
13. Elute the DNA in 50 μL of TE buffer and store at −20 ˚C until further analysis.

Inhibition test

Inhibition of PCR reactions by eDNA samples was assessed using a previously described method [16], which involved targeting the mitochondrial cytochrome b (cytb) gene of the exotic temperate species, *Oncorhynchus masou*, that is known not to occur within the tropical Porong estuaries, as an internal positive control (IPC). For the inhibition assay, a total volume of 20 μL mixture was prepared, comprising 1 × Luna® Universal Probe qPCR Master Mix (#M3004, New England Biolabs, Ipswich, MA, USA), 0.5 μM of each forward and reverse primer, 2 μL of IPC template (4.8×10^4 copy numbers), and 2 μL of extracted eDNA from each water sample. PCR conditions for the inhibition assay involved an initial denaturation at 95 ˚C for 5 min, followed by 40 cycles of denaturation at 95 ˚C for 20 s, annealing at 63.6 ˚C for 20 s, and extension at 72 ˚C for 20 s. The positive control was performed using nuclease-free water, replacing the eDNA samples. Inhibition was indicated by a delayed Cq value (ΔCq), which was calculated by subtracting the Cq value of the sample from that of the positive control. A pairwise Wilcoxon rank-sum test was performed using the rstatix package v0.7.1 [20] to determine differences in ΔCq between two protocols in freshwater and brackish samples.

Metabarcoding library preparation and high throughput sequencing

We employed the MiFish primer set to amplify fish eDNA from the 12S region [21]. Each of eDNA subsamples was used as an individual template for the initial round of PCR library preparation. For the first PCR, a 20 μL reaction mixture was prepared, comprising 2.0 μL of template, 1.0 μL each of 10 pmol forward and reverse primers with adapters, 2.0 μL of 2.5 mM dNTPs, 2.0 μL of 10X EX Taq buffer, 0.2 μL of Ex Taq HS DNA Polymerase (Takara Bio Inc., Shiga, Japan), 0.6 μL of 3 % DMSO, and 11.20 μL of deionized water. The PCR conditions comprised an initial denaturation step at 95 ˚C for 3 min, succeeded by 35 cycles of denaturation at 94 ˚C for 20 s, annealing at 60 ˚C for 15 s, and extension at 72 ˚C for 15 s, with a final extension step at 72 ˚C for 5 min. Negative controls using deionized water as a template were included in the PCR setup. PCR products of approximately 300 bp were purified using the AccuPrep® Gel Purification Kit (Bioneer, Republic of Korea) and subsequently employed as a template for the second indexing PCR using the Nextera XT index kit (Illumina, San Diego, CA, USA). Following purification, the second amplicons of three subsamples from each site were pooled for the respective protocols and the quality and quantity were evaluated using the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). Subsequently, high-throughput sequencing was conducted on the 2 × 300 bp paired-end Illumina MiSeq platform.

Table 2
Bioinformatic summary of raw reads processing. Nonchim – chimeric sequences removal.

Sample	input	filtered	denoisedF	denoisedR	merged	nonchim
Qiagen-freshwater	129,055	127,478	126,638	126,961	124,531	120,823
Qiagen-brackish	151,368	149,285	149,112	149,045	145,888	139,626
GuHCl -brackish	107,800	106,230	105,925	105,863	103,224	87,674
GuHCl -freshwater	173,098	170,643	169,929	170,308	168,183	160,771

Bioinformatics processing and data analysis

The initial steps involved demultiplexing the raw reads, followed by trimming of adapters and index, as well as the elimination of low-quality sequences (QV <20) utilizing the CLC Genomics Workbench v8.0 (CLC Bio, Cambridge, MA, USA). Subsequent removal of primer sequences was conducted using Cutadapt v1.9.1 [22]. The processed reads were denoised to generate Amplicon Sequence Variants (ASVs) using the Divisive Amplicon Denoising Algorithm 2 (DADA2) v1.16.1 [23] and then curated by employing the LULU v0.1.0 algorithm [24]. Taxonomic classification of the curated ASVs was conducted by BLAST v2.10.1+ against the NCBI nucleotide database (accession date: 04/02/2021), complemented by SEED v2.1.1a [25] for placement of higher rank classification ranging from family to kingdom level.

Phyloseq v1.32 [26] and microeco v 0.12 [27] were used to analyze the species composition obtained from each protocol. To ensure uniformity in sequencing depth across samples, the read counts were rarefied to the lowest number of sequences observed in across all samples (87,674). The relative read abundance (RRA) of the freshwater and brackish community samples were pooled by protocol method prior to calculation of the Spearman correlation to facilitate a comparative analysis between the performance of the Qiagen and GuHCl extraction protocols. All data visualizations were generated based on the ggplot2 package v3.3.3 [28] in R v3.6.3 [29]. A web-based Venn diagram tool available at http://bioinformatics.psb.ugent.be/webtools/Venn/ was used for analysis of shared species between protocols.

Method validation

The environmental DNA (eDNA) metabarcoding was successfully conducted using the MiFish universal primer to detect fish communities from filtered water subjected to both protocols. The bioinformatic processing of eDNA metabarcoding data involved several key steps (Table 2). Initial input reads varied across samples, with a range of 107,800 to 173,098 reads. The final step involved the removal of chimeric sequences, yielding a reduction in reads by approximately 6 % (Qiagen-freshwater) to 19 % (GuHCl -brackish) from the input reads.

We identified a total of nine orders, 21 families, 30 genera, and 41 species within the Actinopteri class, and no non-fish sequences were detected in all samples. The detected orders included Beloniformes, Perciformes, Gobiiformes, and others, showcasing the taxonomic diversity captured by this metabarcoding approach. Compared to traditional methods [30], eDNA metabarcoding can detect a wide range of species from a single sample, providing a comprehensive snapshot of the biodiversity in an area. By comparing both protocols, we found that the GuHCl extraction protocol identified a greater number of fish species (n = 20) in freshwater samples than the Qiagen protocol (n = 9) (Fig. 1A). No species were uniquely detected by the Qiagen protocol in freshwater samples, whereas eleven species were exclusively identified by the GuHCl extraction, namely *Systomus orphoides, Channa striata, Liza subviridis, Aplocheilus panchax, Barbonymus schwanenfeldii, Oryzias javanicus, Notopterus notopterus, Anematichthys apogon, Mystus gulio, Gambusia affinis,* and *Gobiopterus chuno* (Table 3). In contrast, the Qiagen protocol identified more fish species in brackish samples (n = 24) compared to the GuHCl protocol (n = 16) (Fig. 1B). No species were uniquely detected by the GuHCl protocol in brackish samples, while eight species were exclusively identified by the Qiagen protocol, including *Leiognathus* sp., *Strongylura* sp., *Cynoglossus* sp., *Dermogenys collettei, Gerres microphthalmus, Trypauchen vagina, Lutjanus johnii,* and *Ambassis gymnocephalus*. The exclusively detected species by respective protocols were low in abundance, ranging from 0.008 % to 0.137 % of RRA (Table 3).

We detected a total of four alien species (*G. affinis, Pterygoplichthys pardalis, Oreochromis niloticus,* and *Oreochromis* sp.) using the GuHCl protocol. On the other hand, the Qiagen protocol detected three of these but failed to detect *G. affinis*. In addition, the GuHCl

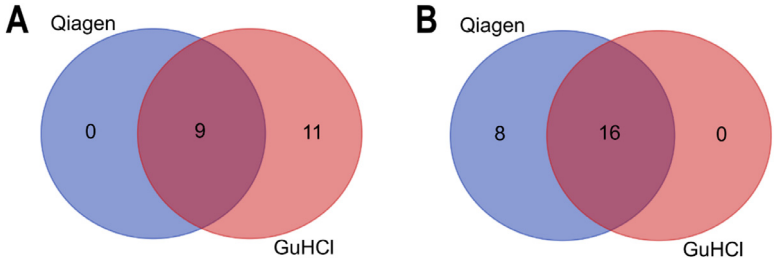

Fig. 1. Venn diagram showing the numbers of shared and exclusive detection of fish species between the two extraction protocols from (A) freshwater and (B) brackish samples.

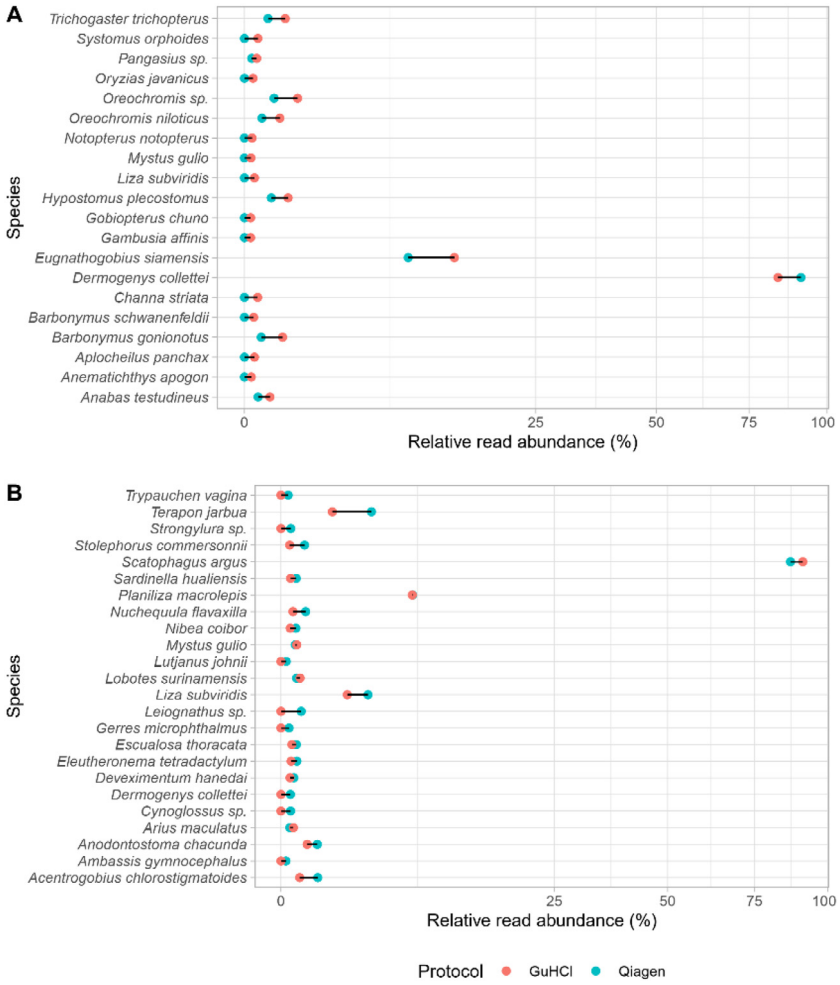

Fig. 2. Dumble plot showing the variability of the relative read abundance of detected fish between two environmental DNA extraction protocols from (A) freshwater and (B) brackish samples.

Table 3
The relative read abundance (%) of exclusively detected species in freshwater and brackish samples by respective protocol.

Fish species	Sample	Qiagen	GuHCl
Systomus orphoides	freshwater	0	0.054
Channa striata	freshwater	0	0.051
Liza subviridis	freshwater	0	0.030
Aplocheilus panchax	freshwater	0	0.030
Barbonymus schwanenfeldii	freshwater	0	0.025
Oryzias javanicus	freshwater	0	0.022
Notopterus notopterus	freshwater	0	0.017
Anematichthys apogon	freshwater	0	0.014
Mystus gulio	freshwater	0	0.013
Gambusia affinis	freshwater	0	0.011
Gobiopterus chuno	freshwater	0	0.011
Leiognathus sp.	brackish	0.137	0
Strongylura sp.	brackish	0.032	0
Cynoglossus sp.	brackish	0.031	0
Dermogenys collettei	brackish	0.030	0
Gerres microphthalmus	brackish	0.022	0
Trypauchen vagina	brackish	0.017	0
Lutjanus johnii	brackish	0.009	0
Ambassis gymnocephalus	brackish	0.008	0

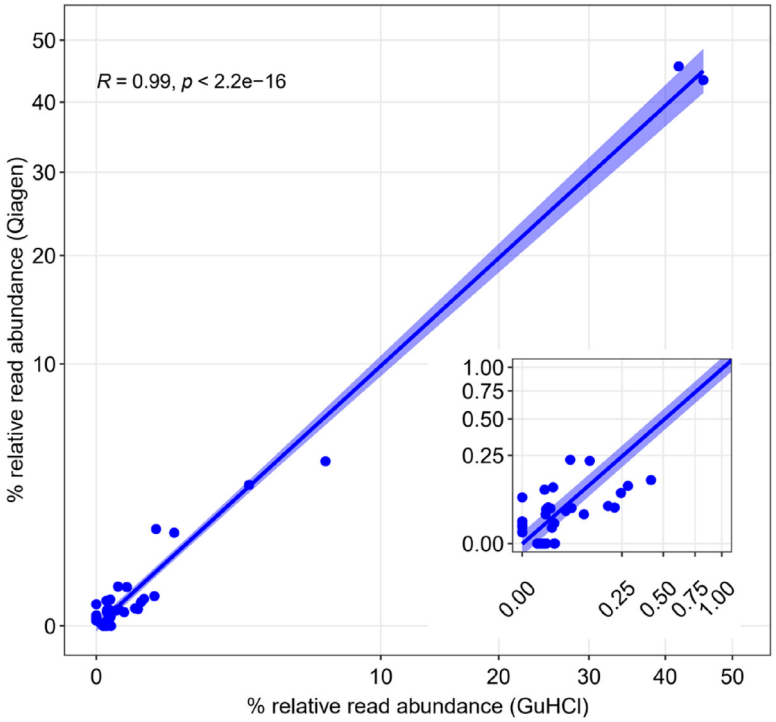

Fig. 3. Spearman correlation of relative read abundance between two environmental DNA extraction protocols.

protocol detected *Notopterus notopterus*, a featherback fish protected by the Ministry of Marine Affairs and Fisheries of Republic of Indonesia; this species was not however detected using the Qiagen kit.

The ability to detect low-abundance fish species is critical in eDNA studies for proactive management, conservation, and biosecurity. In terms of invasive species, early detection is essential for preventing the establishment and spread of low-abundance non-native species, thereby protecting biodiversity and mitigating ecological and economic impacts. Notably, *G. affinis*, an invasive species native to Central and North America and among the world's most widespread invasive freshwater fish [31], was detected by the GuHCl protocol but not by the Qiagen kit. This species is found in the Porong River, a subsystem of the Brantas River [32]. On the other hand, given the typically low populations of protected species, the sensitivity of eDNA methods to detect these species at low abundance is vital for effective conservation, legal compliance, habitat protection, and improving our understanding of their ecology. The GuHCl protocol exclusively detected *N. notopterus*, another low abundance species listed as least concern by the IUCN [33] but is legally protected in Indonesia due to overexploitation and habitat destruction [34]. Since freshwater ecosystems are characterized by high levels of endemism [35] and are particularly susceptible to invasions [36], the GuHCl DNA extraction protocol offers advantages for detecting both invasive and endemic species, as evidenced by the higher species detection rate in freshwater samples.

The RRA of detected fish species was similar between the two protocols, with only five species exhibiting differences > 1 % in RRA (Fig. 2). Specifically, *Eugnathogobius siamensis* and *Scatophagus argus* exhibited 5.07 % and 4.23 % higher RRA with the GuHCl protocol, respectively. On the other hand, *Dermogenys collettei, Terapon jarbua,* and *Liza subviridis* (in brackish sample) demonstrated a 7.39 %, 1.85 %, and 1.06 % higher RRA with the Qiagen protocol, respectively. The Pearson test showed significant correlation ($R = 0.99$, $p < 0.0001$) between generated RRAs from the two protocols (Fig. 3).

Several eDNA metabarcoding studies have utilized presence-absence data to assess fish communities [3] due to the susceptibility of read counts to various influencing factors, including laboratory techniques [37]. However, the correlation between species biomass and read counts in MiFish metabarcoding suggests the feasibility of estimating relative species biomass through sequencing data [38]. Given that both protocols demonstrate comparable results in fish species detection, future assessment of the GuHCl protocol can include feasibility studies of employing RRA reads to determine species biomass in freshwater ecosystems.

Overall, no statistically significant differences were detected in ΔCq values between the GuHCl and Qiagen protocols for both freshwater ($\Delta Cq_{GuHCl} = 0.110 \pm 0.131$ vs $\Delta Cq_{Qiagen} = 0.341 \pm 0.241$) and brackish samples ($\Delta Cq_{GuHCl} = 1.88 \pm 2.24$ vs $\Delta Cq_{Qiagen} = 0.322 \pm 0.189$) (Fig. 4). However, the GuHCl protocol demonstrated slightly greater amplification delays in brackish samples compared to the Qiagen protocol, with a wider range of ΔCq values (ranging from 0.176 to 5.06).

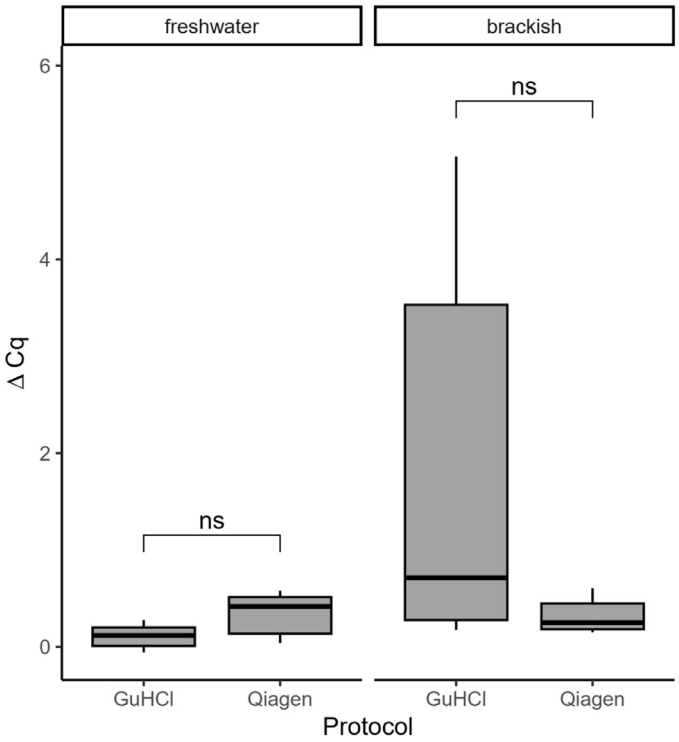

Fig. 4. Inhibition test (ΔCq) of environmental DNA extracted from freshwater and brackish samples using GuHCl and Qiagen protocol.

Limitations

While our GuHCl method detected more fish species in freshwater samples, fewer species were identified in brackish samples compared to the Qiagen kit, likely due to PCR inhibition as observed in this study. Our findings indicate a relatively higher degree of PCR inhibition in brackish samples using GuHCl protocol, evidenced by a greater delay in Ct values. It remains unclear what caused the PCR inhibition in the brackish sample. Given that the brackish samples were obtained from a mangrove area, it is likely that the water samples contained a substantial amount of tannins due to the significant presence of mangrove litterfall [39]. Natural PCR inhibitors such as humic, fulvic, and tannic acids, commonly found in environmental samples, can impede DNA polymerase activity during library preparation [40]. Furthermore, the higher salinity in brackish samples may also contribute to the PCR inhibition, as elevated salt levels have the potential to impede the amplification capacity of thermostable DNA polymerases [41]. This aligns with previous research showing a positive correlation between PCR inhibition and salinity in water samples [16]. However, PCR inhibition of Qiagen kit showed consistent results in freshwater and brackish samples, presenting relatively small delayed Cq values. The presence of these inhibitors can prevent the amplification of low-abundance sequences, as more abundant sequences are preferentially amplified. Moreover, the selection of PCR reagents significantly impacted the extent of inhibition observed [42]. Despite the complexity of natural PCR inhibitors, their impact can be mitigated by employing inhibitor-resistant polymerases [43] and/or incorporating dimethyl sulfoxide (DMSO) into the initial PCR reaction, which acts as a PCR enhancer [44]. Consequently, choosing appropriate PCR reagents is essential to minimize inhibition during library preparation for high-throughput sequencing.

Ethics statements

No ethical approvals or considerations were necessary for this study.

CRediT author statement

Muhammad Hilman Fu'adil Amin: Conceptualization, Methodology, Software, Data curation, Funding acquisition, Writing- Original draft, Writing- Reviewing and Editing. **Hyun-Woo Kim:** Methodology, Resources, Writing- Original draft. **Amy Yee-Hui Then:** Funding acquisition, Data curation, Writing- Reviewing and Editing. **Nur Indradewi Oktavitri:** Data curation, Writing - Original draft. **Ah Ran Kim:** Methodology, Formal analysis. **Soo Rin Lee:** Methodology, Visualization. **Manikya Pramudya:** Formal analysis, Project administration. **Sapto Andriyono:** Formal analysis, Visualization. **Annisa Selvia Widyar Iswara:** Formal analysis, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw reads were available in GenBank under BioProject PRJNA1100780 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1100780).

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