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Development of species identification techniques for anguillid eels using species-specific genetic markers

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The decline in eel resources, coupled with the challenges of morphological identification and the rise of illegal trade, highlights the urgent need for accurate species identification techniques. To address this, a multiplex PCR assay was developed, targeting the mitochondrial Cytochrome b gene. Species-specific primers were designed and their efficacy validated through single PCR. The multiplex PCR conditions were then optimized to enable the simultaneous amplification of five major eel species. This assay exhibited high accuracy, specificity, and sensitivity, successfully identifying all five species even when DNA concentrations were low. This multiplex PCR assay offers a rapid and cost-effective solution for eel species identification, with the potential to significantly bolster eel conservation efforts. By enabling accurate species identification, it can help combat illegal trade and support the sustainable management of eel resources.

KEYWORDS

***anguilla*, multiplex PCR, species identification, Cytochrome b, species-specific markers**

1 Introduction

Anguillid eels (often called freshwater eels), with their wide global distribution, hold significant ecological and economic importance (Jacoby et al., 2015). Their unique catadromous life cycle, coupled with their high economic value as a delicacy in East Asian countries like Korea, Japan, and China, has led to intense fishing pressure and concerns about their sustainability (Tsukamoto and Arai, 2001; Yuan et al., 2022). Eels are catadromous fish, meaning they migrate from freshwater to saltwater to spawn (Tsukamoto and Arai, 2001). The hatched larvae then embark on a long journey back to rivers, aided by ocean currents, where they mature into adults (Durif et al., 2022). This complex life cycle poses a significant challenge for effective eel resource management.

In recent years, eel resources have been rapidly declining due to a combination of factors, including overfishing, habitat destruction, marine pollution, climate change, and dam construction (Drouineau et al., 2018). This decline has raised international concerns, underscored by the listing of the Japanese eel (*Anguilla japonica*) as an endangered species on the International Union for Conservation of Nature (IUCN) Red List in 2014, and the European eel (*A. anguilla*) in 2020 (Jacoby and Gollock, 2014; Pike et al., 2020). These circumstances necessitate urgent and concerted efforts to effectively manage and conserve eel resources (Jacoby et al., 2015; Righton et al., 2021). The IUCN Red List, while providing crucial information on species status, does not inherently possess regulatory mechanisms. However, it serves as a critical foundation for conservation efforts, including the implementation of international agreements such as the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

CITES plays a vital role in regulating international trade in endangered species, including Anguillid eels, to ensure their survival. The accurate identification of eel species is essential for CITES implementation, particularly for monitoring trade, enforcing regulations, and preventing illegal trafficking. The morphological identification of eel species, particularly challenging at the glass eel stage, has led to serious problems such as illegal fishing and smuggling, as well as origin fraud (Stein et al., 2016; Alonso and van Uhm, 2023). These issues not only pose significant obstacles to eel resource management but also disrupt the ecological balance, further increasing the risk of extinction for some species (Stein et al., 2021). This problem extends beyond glass eels to eel products in the food industry, where mislabeling and species substitution can occur (Goymer et al., 2023). Accurate species identification is crucial for combating fraud and ensuring the legality and sustainability of the eel trade. In this respect, DNA analysis can be a valuable tool for identifying the species and origin of eel products, contributing to the conservation efforts and responsible management of eel resources (Noh et al., 2018).

Therefore, the development of accurate and rapid eel species identification techniques is crucial for the sustainable use and conservation of eel resources. However, most existing eel species identification techniques have limitations. Single PCR assays can only identify one species at a time, while methods such as real-time PCR or PNA probe assays involve high costs, complex procedures, and the need for skilled personnel and expensive equipment (Sezaki et al., 2005; Itoi et al., 2005; Noh et al., 2018). While semi-multiplex PCR has been employed for eel species identification, it has been observed to produce non-specific amplification in addition to species-specific identification (Fahmi et al., 2013). These limitations hinder both the field applicability and the efficiency of eel species identification.

This study aimed to address these challenges by developing a multiplex PCR assay capable of accurately and efficiently identifying five commercially important eel species imported into Korea: Japanese eel (*A. japonica*), American eel (*A. rostrata*), Indonesian shortfin eel (*A. bicolor pacifica*), European eel (*A. anguilla*), and giant mottled eel (*A. marmorata*) (Noh et al., 2018). These eels are imported both live and as processed fisheries products. Live eels, primarily glass eels, are imported for aquaculture purposes, while processed fisheries products include various forms such as fresh, frozen, and processed eels. The developed assay utilizes markers that specifically bind to and amplify species-specific regions within the mitochondrial Cytochrome b (Cytb) gene (Minegishi et al., 2005).

This approach offers several advantages, including simplicity, cost-effectiveness, and easy visualization of results through gel electrophoresis (Matsunaga et al., 1999). Therefore, the findings of this study are expected to have broad applicability in various fields related to eel resource management and conservation, including on-site eel species identification, quarantine inspection of imported eels, authenticity verification of eel products, and the prevention of illegal fishing and trade (Gill, 2007; Espiñeira and Vieites, 2016), ultimately contributing to the establishment of a science-based management system for the sustainable use and conservation of eel resources, the maintenance of ecological balance, and the effective implementation of international regulations like CITES.

2 Materials and methods

2.1 Gene sequence acquisition and primer design

In this study, mitochondrial Cytb gene sequences for five eel species (*A. japonica*, *A. rostrata*, *A. bicolor pacifica*, *A. anguilla*, and *A. marmorata*) were obtained from the NCBI GenBank database (Supplementary Table S1). Multiple sequences per species were collected to account for intraspecific genetic variation. The obtained sequences were aligned using MegAlign Pro v17.3.0 software (DNASTAR Lasergene, Madison, WI, USA), and interspecific sequence differences were analyzed to identify species-specific regions using DnaSP v5.10.01 software (Librado and Rozas, 2009). Based on these regions, a universal reverse primer (binding to all five eel species) and species-specific forward primers were designed, with special consideration given to placing the species-specific nucleotide variations at the 3' end of the primers to enhance specificity (Kang et al., 2015). Primer design also considered specificity, amplicon size, Tm value, and avoidance of secondary structure formation to enhance PCR efficiency and species identification accuracy (Dieffenbach et al., 1993). The designed primers were synthesized by Genotech Corporation (Seoul, Korea).

2.2 Sample collection and DNA extraction

Muscle tissues were obtained from 42 eel samples, with up to 10 individuals per species, from the National Institute of Fisheries Science's Fisheries Bio-Resources Storage (Busan, Korea) (Table 1). Each individual was an accurately identified adult specimen to ensure the reliability of the results. The collected tissue samples were incubated with 8M TNES-UREA solution and Proteinase K at 37°C for 12 hours for protein digestion and cell lysis (Chomczynski and Sacchi, 1987). Genomic DNA was then extracted using the

TABLE 1 *Anguilla* species specimens used in this study.

No.	Species	Number of samples	Specimen accession number
1	*Anguilla japonica*	10	NFRDI-FI-TS-0073097~0073106
2	*Anguilla rostrata*	2	NFRDI-FI-TS-0077939~0077940
3	*Anguilla bicolor pacifica*	10	NFRDI-FI-TS-0075207~0075216
4	*Anguilla anguilla*	10	NFRDI-FI-TS-0073157~0073166
5	*Anguilla marmorata*	10	NFRDI-FI-TS-0073247~0073256

phenol-chloroform extraction and ethanol precipitation methods (Wasko et al., 2003). The purified DNA was quantified and its purity assessed using a NanoPhotometer N60 (IMPLEN, Munich, Germany). Only high-quality DNA samples, free of PCR inhibitors, were used for further experiments. The purified DNA samples were stored at -20°C until further use.

2.3 Single PCR and validation

To validate the specificity and sensitivity of the designed primers, single PCR amplifications were conducted using genomic DNA samples from each eel species. Single PCR is a technique to amplify a specific target DNA sequence using a single pair of primers. Gradient PCR was performed to determine the optimal annealing temperature for species-specific primer binding. The annealing temperature was systematically varied across a range of 50-65°C in a single PCR reaction. PCR reactions were prepared using a 2X PCR master mix (iNtRON Biotech, Seoul, Korea) according to the manufacturer's instructions. A typical PCR master mix contains Taq DNA polymerase, dNTPs, MgCl2, and reaction buffer. Each PCR reaction contained 1 µL of template DNA, 1 µL of each primer (10 pmol), 10 µL of 2X PCR master mix, and 7 µL of distilled water, resulting in a final volume of 20 µL. The PCR amplification was performed using an VeritiTM thermal cycler (Applied Biosystems, CA, USA) under the following conditions: an initial denaturation at 94°C for 7 minutes, followed by 34 cycles of denaturation at 94°C for 30 seconds, annealing at 50-65°C (gradient) for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes. Amplified PCR products were visualized on a 1.5% agarose gel and imaged using a GelDoc Go system (BioRad, Hercules, CA, USA). Subsequently, the PCR products were finally verified by sequencing analysis. Sequencing was performed using an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and the species were identified using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Multiplex PCR and validation

PCR amplicon sizes for each eel species were confirmed, and multiplex PCR conditions (primer concentration and annealing temperature) were optimized stepwise to ensure efficient and specific amplification. The PCR reaction mixture contained template DNA, five species-specific primer sets, and a commercial PCR master mix. PCR products were visualized by gel electrophoresis (1.5% agarose gel) and stained with Loading star (DyneBio, Daejeon, Korea). The initial primer concentration was set at 0.3 µL (10 pmol) per primer and was adjusted based on the amplification results to achieve optimal conditions. The annealing temperature was optimized to ensure the specific binding of all five primers. To determine the sensitivity of the selected primer sets, DNA samples from each eel species were diluted to concentrations of 10 ng/µL, 1 ng/µL, 0.1 ng/µL, and 0.01 ng/µL, and then analyzed under the optimized conditions. The PCR conditions for this analysis were identical to those used in the single PCR.

3 Results

3.1 Mitochondrial Cytb gene sequence and haplotype analysis

Analysis of the complete 1,140 bp mitochondrial Cytb gene sequences from a total of 281 individuals representing five eel species (obtained from NCBI GenBank) revealed that each species possessed various haplotypes, with 276 nucleotide variations observed across all individuals. While there was variation within each species, distinct sequence differences were consistently observed between species, allowing for clear differentiation. The Japanese eel (*A. japonica*) exhibited 30 haplotypes, including seven that were unique sequence variations. The American eel (*A. rostrata*) had 29 haplotypes, including four that were unique sequence variations. The Indonesian shortfin eel (*A. bicolor pacifica*) exhibited 26 haplotypes, including 14 that were unique sequence variations. The European eel (*A. anguilla*) had 48 haplotypes, including two that were unique sequence variations. Finally, the giant mottled eel (*A. marmorata*) had 97 haplotypes, with seven unique sequence variations.

3.2 Primer design and single PCR validation

Based on the identified species-specific sequence variations, a universal reverse primer and species-specific forward primers were designed. Species-specific primers were designed based on the identified unique sequence variations, prioritizing *A. anguilla*, which exhibited the fewer unique variations. The primer for *A.*

anguilla was designed to target a T nucleotide at position 329 bp (A in other species) at its 3' end. Next, a primer for *A. rostrata* was designed, targeting a C nucleotide at position 510 bp (T in other species). Subsequently, the *A. marmorata*-specific primer was designed to target a T nucleotide at position 654 bp (C in other species). For A. japonica, the primer targeted a C nucleotide at position 115 bp (T in others), and finally, the primer for *A. bicolor pacifica* targeted an A nucleotide at position 789 bp (C or T in other species). A universal reverse primer was also designed to bind to a conserved region in the mitochondrial genome downstream of the Cytb gene, ensuring its compatibility with all five eel species (Table 2, Figure 1).

To validate the specificity and sensitivity of the designed primers, single PCR amplifications were conducted using genomic DNA samples from each eel species. Single PCR is a technique to amplify a specific target DNA sequence using a single pair of primers. Gradient PCR was performed to determine the optimal annealing temperature for species-specific primer binding. The annealing temperature was systematically varied across a range of 50-65°C in a single PCR reaction. Gradient PCR analysis revealed that an annealing temperature of 62°C resulted in species-specific binding and amplification for all selected primers. Single PCR assays using these primers on DNA samples from each species yielded PCR amplicons of the expected sizes (Japanese eel: 1,119 bp,

TABLE 2 List of species-specific genetic markers based on sequence variations.

Primer name	Target species	Sequence (5' to 3')	Tm(°C)
Ajap-115	*Anguilla japonica*	(F) TTTTGGCTCTCTCCTAGGAC	63
Aros-510	*Anguilla rostrata*	(F) TGAGGGGGCTTTTCAGTC	65
Abip-789	*Anguilla bicolor pacifica*	(F) CCCGGCAAATCCTATAGTA	61
Aang-329	*Anguilla anguilla*	(F) TTTACTACGGCTCATACCTTTACAT	63
Amar-654	*Anguilla marmorata*	(F) CGACGCGGACAAAATT	60
Auni-1213	Universal	(R) AGCGCTAGGAAGAATTTTAATC	60

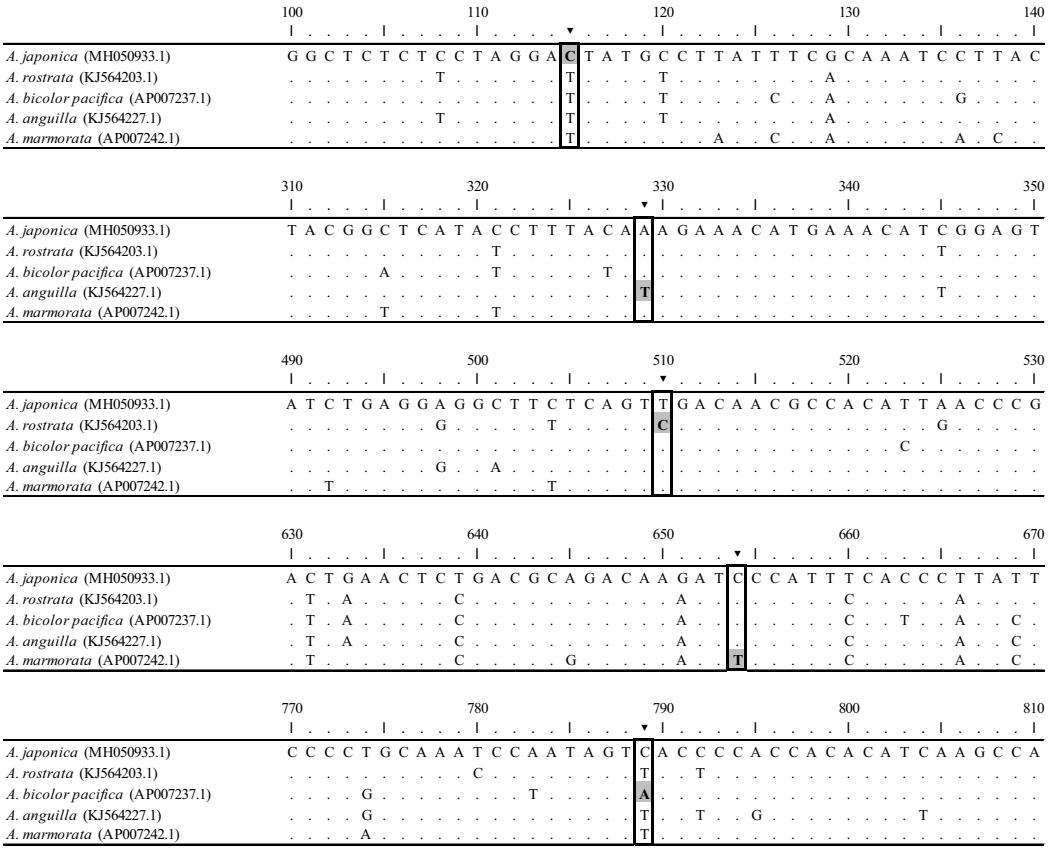

FIGURE 1
Species-specific sequence variations in the mitochondrial Cytb region of *Anguilla* species.

European eel: 910 bp, American eel: 722 bp, giant mottled eel: 576 bp, Indonesian shortfin eel: 444 bp), confirming primer specificity (Figure 2). Sequencing of these amplicons confirmed their identity, with each sequence perfectly matching that of the corresponding target species.

3.3 Multiplex PCR for eel species identification

To optimize the assay for simultaneous identification of all five eel species, optimal conditions were explored by mixing all five primers together. Based on the optimal temperature of 62°C determined in single PCR, the primer concentration was gradually increased from a minimum of 0.3 µl (10 pmol) to ensure clear interpretation of PCR amplification results. It was found that *A. bicolor pacifica* and *A. marmorata* required approximately three times higher primer concentrations than other species. In a final reaction volume of 20 µl, the optimal primer concentrations were determined to be 0.3 µl (10 pmol) for *A. japonica*, *A. rostrata*, and *A. anguilla*, and 1 µl (10 pmol) for *A. bicolor pacifica* and *A. marmorata* (Table 3). The optimized multiplex PCR conditions yielded clear results, allowing for the successful differentiation of all five eel species (Figure 3).

Furthermore, when DNA from all five eel species was mixed and analyzed, the species-specific PCR products were clearly separated on a 1.5% agarose gel, with no interference or cross-reactivity. The assay accurately identified all 42 eel samples (up to 10 individuals/species) based on expected amplicon patterns. Sensitivity evaluation using diluted DNA samples showed successful detection of all five species even at 0.1 ng/µL, confirming the robustness of the method (Figure 4).

4 Discussion

This study successfully developed a multiplex PCR assay for accurate identification of five major eel species. Through the analysis of mitochondrial Cytb gene sequences and identification of species-specific sequence variations, a set of primers was designed that enable precise identification of these five eel species within a single PCR reaction. The assay demonstrated high specificity and sensitivity, overcoming the limitations of single PCR assays and the high cost/complexity of real-time PCR or PNA probe assays. This represents a significant advancement in the field of eel species identification, particularly considering the challenges associated with morphological identification, especially at the glass eel stage.

The use of the mitochondrial Cytb gene as a genetic marker aligns with previous research demonstrating its effectiveness in species discrimination due to its high variability and maternal inheritance (Sezaki et al., 1999; Aoyama et al., 2001; Fahmi et al., 2013; Noh et al., 2018). A multiplex PCR system based on species-specific primers was established, and a comprehensive analysis of the complete Cytb gene sequences available in public database was conducted to identify species-specific sequence variation for primer design. This approach offers several distinct advantages over existing methods, including its simplicity, cost-effectiveness, and straightforward visualization of results through gel electrophoresis, rendering it highly suitable for field applications and resource-limited settings (Kim et al., 2023).

This assay can simplify and aid monitoring and enforcement efforts, particularly in combating illegal fishing and trade of endangered species (Jaser et al., 2021). Specifically, this assay can directly support the implementation of CITES by providing a reliable tool for monitoring international trade in Anguillid eels. By enabling accurate species identification, it can help to detect illegal trafficking, ensure that trade is conducted in accordance with CITES regulations, and prevent illegal fishing and smuggling of glass eels, thereby safeguarding valuable eel resources. Moreover, this assay can contribute to the establishment of science-based management system for sustainable eel resources by providing accurate species identification data for monitoring populations, assessing genetic diversity, and informing conservation strategies. Furthermore, the assay's ability to verify the authenticity of eel products enhances consumer confidence and promotes fair trade practices, which are crucial for sustainable eel aquaculture and market regulation (Espiñeira and Vieites, 2016).

Beyond its immediate impact on eel conservation, this study highlights the power of molecular tools in addressing critical challenges in biodiversity conservation and resource management. The development of similar multiplex PCR assays for other taxa, particularly those facing threats from overexploitation or illegal trade, could significantly enhance our capacity for effective monitoring and enforcement. While this study focused on five

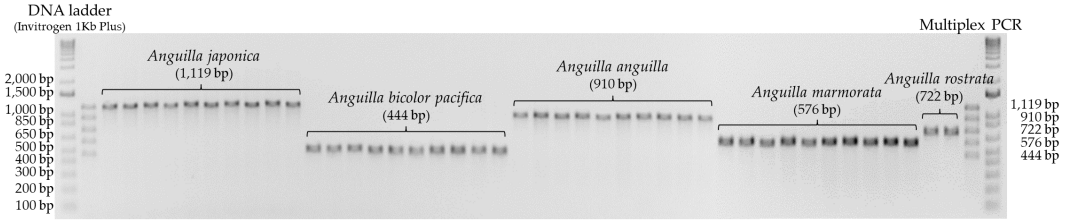

FIGURE 2
Results of single PCR amplification using species-specific primers for five *Anguilla* species.

TABLE 3 Optimized multiplex PCR reaction composition.

Component	Volume (μl)
Template DNA	1.0
Forward primer	2.9
Ajap-115	(0.3)
Aros-510	(0.3)
Abip-789	(1.0)
Aang-329	(0.3)
Amar-654	(1.0)
Reverse primer	1.5
Auni-1213	(1.5)
2X PCR master mix	10.0
Distilled water	3.6
Total	20

commercially important eel species in Korea, the assay's applicability could be expanded in future research by incorporating primers for additional species, particularly those that are threatened or play crucial ecological roles. This would enhance the assay's utility for broader biodiversity monitoring and conservation efforts. Moreover, the integration of this assay with emerging technologies like environmental DNA (eDNA) analysis and portable sequencing platforms could further revolutionize our approach to biodiversity assessment and conservation (Cho et al., 2016; Farley et al., 2018).

Further improvements could involve simplifying DNA extraction procedures, potentially through the use of commercial kits or non-invasive sampling methods such as collecting DNA with swabs or eDNA, and refining primer design for even faster and more efficient analysis (Mason and Botella, 2020). The development of portable, field-deployable devices such as microfluidic platforms that integrate DNA extraction and multiplex PCR analysis would greatly enhance the assay's on-site utility for rapid species identification and monitoring (Khodakov et al., 2021).

5 Conclusion

This study presents a significant advancement in eel resource management and conservation by developing a multiplex PCR assay capable of rapid and accurate identification of five major eel species. The design of species-specific primers based on the mitochondrial Cytb gene ensures high specificity and sensitivity, overcoming the limitations of existing methods. In particular, the accurate identification of species at the glass eel stage can prevent illegal fishing and smuggling, contributing to the establishment of a scientific management system for the sustainable use of eel resources.

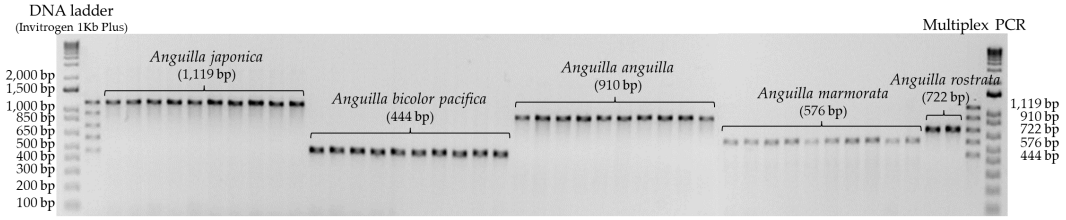

FIGURE 3
Results of multiplex PCR amplification using species-specific primers for five *Anguilla* species.

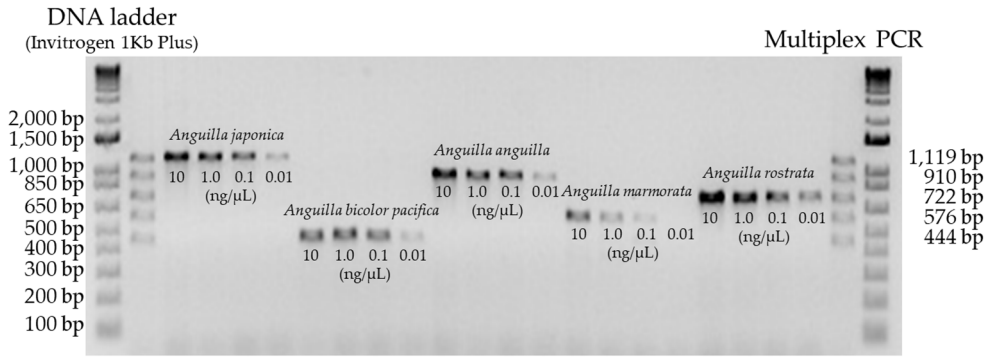

FIGURE 4
Sensitivity evaluation of the multiplex PCR assay for five *Anguilla* species using different DNA concentrations (10 ng/μL, 1.0 ng/μL, 0.1 ng/μL, and 0.01 ng/μL).

The approach developed in this study has the potential to be applied to the conservation and management of not only eels but also other endangered or overfished species. Future expansion of the assay's scope and technological improvements are expected to make even greater contributions to the field of biodiversity conservation and resource management.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of National Institute of Fisheries Science, Republic of Korea (2024-NIFS-IACUC-2). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

EN: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. CD: Methodology, Writing – review & editing. HP: Software, Writing – review & editing. EK: Conceptualization, Writing – review & editing. HJ: Software, Writing – review & editing. HK: Investigation, Writing – review & editing. YK: Investigation, Writing – review & editing.

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Conflict of interest

The 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.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2025.1518562/full#supplementary-material

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