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Chia-Hsuan Sung
Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan b, chsung@mail.tfrin.gov.tw

Liang-Jong Wang
Forest Protection Division, Taiwan Forestry Research Institute, Taipei, Taiwan

Chang-Wen Huang
Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan, cwhuang@mail.ntou.edu.tw

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RESEARCH ARTICLE

Rapid Species Identification of *Meretrix lusoria* and Three Other *Meretrix* Clams Using PCR and RFLP Analysis of the Mitochondrial COII Gene

Chia-Hsuan Sung [a,b], Liang-Jong Wang [c], Chang-Wen Huang [a,*]

[a] Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan
[b] Technical Service Division, Fisheries Research Institute, Keelung, Taiwan
[c] Forest Protection Division, Taiwan Forestry Research Institute, Taipei, Taiwan

Abstract

***Meretrix* clams are among the most economically important species of bivalves in Eastern Asia and Taiwan. In the past, the identification of these hard clams depended on the shell morphology; however, species identification based on shell markings and shapes confuses the taxonomy of the *Meretrix* genus. The DNA molecular information is useful and can easily identify them accurately and quickly, especially the sequence of mitochondrial DNA. In this study, a PCR method to analyze the mitochondrial cytochrome c oxidase II (COII) gene was developed to rapidly identify of *M. lusoria*, *M. lamarckii*, *M. lyrata* and *M. petechialis*. Four different species of hard clams were easily distinguished by examining the COII PCR gel pattern using the naked eye. The limit of COII PCR detection was 39.06 pg, and it showed good specificity of primer. Further verification using RFLP suggested that four species of hard clams could be identified on the basis of the RFLP gel pattern produced by the digestion of one restriction enzyme, and these results were consistent with the PCR results. The ML phylogenetic analysis of COII grouped the four *Meretrix* species into different clades with strong bootstrap support. These results are consistent with those of the phylogenetic analysis of COI gene. In summary, this study's COII PCR and RFLP method facilitates the rapid, accurate, and inexpensive identification of *Meretrix* species. These results suggest that this COII PCR method can be used for routine and rapid species identification on population, aquaculture and breeding management.**

Keywords: Species identification, *Meretrix*, Hard clams, Cytochrome c oxidase II

1. Introduction

The *Meretrix* clams (Bivalvia: Veneridae) are among the most economically important species of bivalves. These clams are widely distributed along the coasts of East Asia and Southeast Asia, including India, China, Japan, Korea, and Taiwan [1]. According to the Food and Agriculture Organization (FAO) Fishery and Aquaculture Statistics 2019 [2], the primary production area is Eastern Asia, with average annual production and value of farmed hard clams at 55,310.7 tones and 130.4 million US dollars, respectively, from 2010 to 2019. Furthermore, greater than 95% of the production occurred in Taiwan. In the past, the major species of farmed hard clam in Taiwan was *Meretrix lusoria* or *Meretrix petechialis* [3,4]. The species identification of the genus *Meretrix* hard clams were based on the morphological and meristic characteristics of the shell such as the number of hinge teeth, the shape of the shell, the scar of adductor muscle, the pallial sinus and the pallial line [5]. However, species identification based on shell markings and shapes has made the taxonomy of the *Meretrix* genus confusing [1]. The shell form and color of *M. lusoria* and *M. petechialis* are extremely similar, which has

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* Corresponding author.
E-mail addresses: chsung@mail.tfrin.gov.tw (C.-H. Sung), josephwang23@tfri.gov.tw (L.-J. Wang), cwhuang@mail.ntou.edu.tw (C.-W. Huang).

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led to many erroneous identifications and notations [6]. Therefore, the fishmongers could not distinguish which species they sold in the fish markets. Because those characteristics were not always distinguishable, the identification of hard clam species based solely on morphological characteristics was difficult. At present, DNA-based analysis methods are the most useful and reliable tools for species identification and phylogenetic analysis. Folmer et al. [7] described a pair of universal mitochondrial cytochrome c oxidase subunit I gene (COI) primer from 11 invertebrate phyla. These universal primers were also suitable for the identification of *Meretrix* species [8] and other bivalves [9,10]. Usually, the COI gene was the first choice for species identification. In addition to the COI gene, several gene DNA fragments were reported for the identification of bivalve species, such as the internal transcribed spacer (ITS) [11,12], 5S rDNA [13], 16S rDNA [14−16], 18S rDNA [14], adhesive foot protein gene [9], and polyphenolic adhesive protein (PAP) [17,18]. However, DNA sequencing or nucleotide sequence-based analysis is necessary for most genes since the species cannot be rapidly identified from only on their polymerase chain reaction (PCR) electrophoresis results.

The present study aimed to develop a rapid and reliable identification method for four *Meretrix* species (*M. lusoria, Meretrix lamarckii*, *M. lyrata* and *M. petechialis*) using PCR amplification of the mitochondrial COII gene and analysis of the restriction fragment length polymorphism (RFLP) of the amplified amplicons.

2. Materials and methods

2.1. Hard clams sampling, DNA extraction and species identification

Twenty-three specimens of *M. lusoria* were collected from the Shirakawa river in Kumamoto, Japan. Fifteen specimens of *M. lamarckii* were obtained from the coast of Kinmen, Taiwan. Twenty-three specimens *M. petechialis* were purchased from Qingdao, China, and twenty-six specimens of *M. lyrata* were purchased from an online store that imported the clams from Vietnam. The muscle tissues of *Meretrix* clams were stored in ethanol. The total genomic DNA was extracted from the foot muscle using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). All samples of *Meretrix* clams were identified using the COI gene primer set (Table 1) with the following PCR cycle: 94 °C for 2 min, 35 cycles of 94 °C for 30 s/54 °C for 30 s/72 °C for 50 s and finally 72 °C for 10 min. PCR products were purified from gel with the PCR Clean-Up & Gel Extraction Kit (GeneDireX, Inc., USA). The Sanger sequencing was performed by Genomic, Taiwan. Species were identified by BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the nucleotide sequences available in the US National Center for Biotechnology Information (NCBI) GenBank database.

2.2. Design of the COII PCR primers, optimal COII PCR amplification and DNA sequencing

Four mitogenome sequences (*M. petechialis* EU145977 [19], *M. lyrata* NC_022924 [20], *M. lamarckii* GU071281 [21], and *M. lusoria* MT418596 [22]) were downloaded from the NCBI GenBank database and aligned to identify their conserved regions (Fig. 1). The conserved primer of the mitochondrial cytochrome c oxidase II (COII) gene was designed using CLC Genomics Workbench (Qiagen). The DNA sequences of COII PCR primers are shown in Table 1. The basic PCR conditions were 94 °C for 2 min, 30 cycles of 94 °C for 30 s/50 °C for 30 s/72 °C for 50 s and, finally, 72 °C for 10 min. To determine the optimal PCR conditions, various annealing temperatures (48 °C, 50 °C, 52 °C, 54 °C, 56 °C, 58 °C, 60 °C, 62 °C, and 64 °C) and numbers of cycles (26, 28, 30, 32, and 35) were also tested. Each PCR reaction mixture contained 50 ng of DNA template, 10 μL of 2X Ex Taq HS, and 0.4 μL of COII PCR primers (10 μmol/L). The PCRs were conducted in a ProFlex PCR System (Thermo Fisher Scientific Inc., USA). The PCR products were then analyzed

Table 1. The synthetic oligonucleotides used to identify the Meretrix *clams via PCR in this study.*

Target Region	Primer Name	Sequences	Tm	Source
Cytochrome oxidase subunit I (COI)	LCO1490	5′- GGTCAACAAATCATAAAGATATTGG -3′	54	Folmer et al., 1994
	HCO2198	5′- TAAACTTCAGGGTGACCAAAAAATCA -3′		
Cytochrome oxidase subunit II (COII)	Mer_cox2_F265	5′- TTATATGMTATR GAGGTTGG -3′	56	This study
	Mer_cox2_R935	5′- TCTCTAACACCTCACCTATGMAT -3′		
18SrRNA gene	18SF1	5′- GGGTTCGATTCCGGAGAGGGAG -3′	50	Chang et al., 2011
	18SR1	5′- CTTCGAACCTCTRACTTTCGTTCTTGAT -3′		

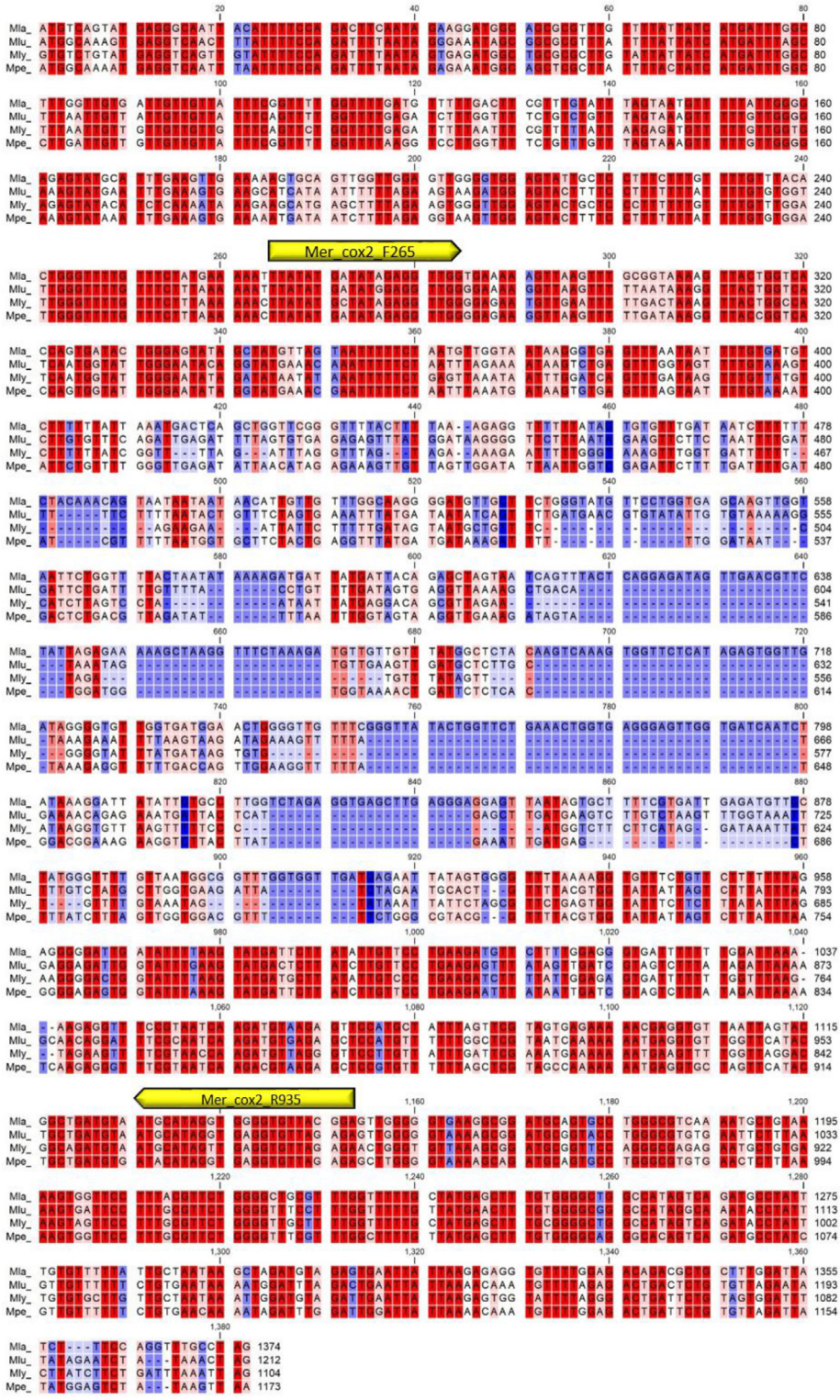

Fig. 1. The COII DNA sequence alignment of four Meretrix clams. Mla: the COII gene of M. lamarckii (GU071281), Mlu: the COII gene of M. lusoria (MT418596), Mly: the COII gene of M. lyrata (NC_022924) and Mpe: the COII gene of M. petechialis (EU145977). The level of sequence conservation is shown on a color scale, with blue residues being the least conserved and red residues being the most conserved. The PCR primer locations are shown by yellow arrows above the alignment.

by 2% agarose gel electrophoresis. The length of the amplicons was obtained using a OneMARK 100 bp DNA marker (GeneDireX, Inc., USA) as a reference. The optimal reaction conditions were determined and used in all further experiments. The PCR products were purified from the gels using a PCR Clean-Up & Gel Extraction Kit (GeneDireX, Inc., USA). Sanger sequencing of the samples was performed by Genomics, Taiwan. The species identification was performed using the BLAST search on the NCBI website against the nucleotide sequences available in GenBank.

2.3. Limit of detection (LOD) and specificity of primer of the COII PCR

For LOD analysis, Two-fold serial dilutions (2.5 ng, 1.25 ng, 625 pg, 312.5 pg, 156.25 pg, 78.13 pg, and 39.06 pg) of the total genomic DNA that was extracted from the *M. lusoria* were used as the template. For primer specificity, the DNA was extracted from common bivalves (*Cyclina sinensis*, *Corbicula fluminea*, *Mercenaria mercenaria*) that were obtained from fish markets in Taiwan. Samples of *Tapes literatus* and *Ruditapes variegata* were obtained from the Taiwan Fisheries Research Institute. Total of 50 ng genomic DNA was used to assess COII PCR primer specificity. The PCR conditions were 94 °C for 2 min, 32 cycles of 94 °C for 30 s/56 °C for 30 s/72 °C for 50 s and, finally, 72 °C for 10 min. The PCR products were mixed with Novel Juice 6 × loading buffer (GeneDireX, Inc., USA) and then observed vai electrophoresis on 2% agarose gel.

2.4. Comparison of the gel pattern of the COI, COII, and 18S PCR products

The COI and COII PCR conditions were described as section 2.3. The 18S PCR primers [23] are shown in Table 1. The PCR conditions were 94 °C for 2 min, 32 cycles of 94 °C for 30 s/50 °C for 30 s/72 °C for 50 s and, finally, 72 °C for 10 min. The PCR products were mixed with Novel Juice 6 × loading buffer and then visualized by e via electrophoresis on 2% agarose gel.

2.5. Verification of the of COII PCR fragments by RFLP analysis

The COII PCR conditions were described as section 2.3. The restriction sites of the COII gene for the four *Meretrix* species were predicted by BioEdit [24]. Five restriction enzymes, *Bgl*II, *Bsp*HI, *Eco*RV, *Pvu*II and *Hpy*188I (NEB, USA), were selected and provided diagnostic restriction pattern for the RFLP analysis of the *Meretrix* hard clams. The digestion conditions were recommended by the manufacturer. The products were mixed with Novel Juice 6 × loading buffer then visualized via electrophoresis on 2% agarose gel.

2.6. Phylogenetic analyses

Phylogenetic relationships were based on the COI and COII gene sequences of four *Meretrix* hard clams. Sequences were aligned using BioEdit software. The best model was selected using Maximum likelihood (ML) method with the Akaike and Bayesian information criterion scores. The Hasegawa-Kishino-Yano (HKY) model with Invariant site (+I) was selected as the most suitable nucleotide substitution model. The ML tree was constructed using MEGA XI [25] with 1000 bootstrap s repeats under model HKY + I model. Four mitogenome sequences (*M. petechialis* EU145977, *M. lyrata* NC_022924, *M. lamarckii* GU071281, and *M. lusoria* MT418596) were used as the reference sequence, the out group was *Mytella strigata*.

3. Results and discussion

3.1. Optimization of the COII PCR reaction conditions

The COII PCR was conducted by using the genomic DNA of *M. lusoria* as a template to determine the optimal temperature and number of cycles for the reaction. Figure 2A shows that the PCR product was observed at 48 °C–64 °C. The amount of PCR products decreased significantly at temperatures greater than 60 °C. The PCR products at 56 °C were slightly stronger than those at 58 °C. Therefore, 56 °C was chosen as the annealing temperature. Conversely, the PCR product was detected at 26, 28, 30, 32, and 34 cycles at 56 °C (Fig. 2B). Based on the gel electrophoresis results, the band pattern stabilized after 32 cycles, and, thus, subsequent experiments were performed using 32 cycles.

3.2. Limit of detection (LOD) and specificity of primer of the COII PCR

In this study, we chose the mitochondrial COII gene as the target fragment for the PCR because the copy number of the mitochondrial DNA is greater than that of the nuclear DNA [26] and is, therefore, conducive to the analysis of the PCR results. In addition, the limitation of the PCR detection is also associated with the various primers, DNA quality, DNA polymerase, and the analytical reagents [27].

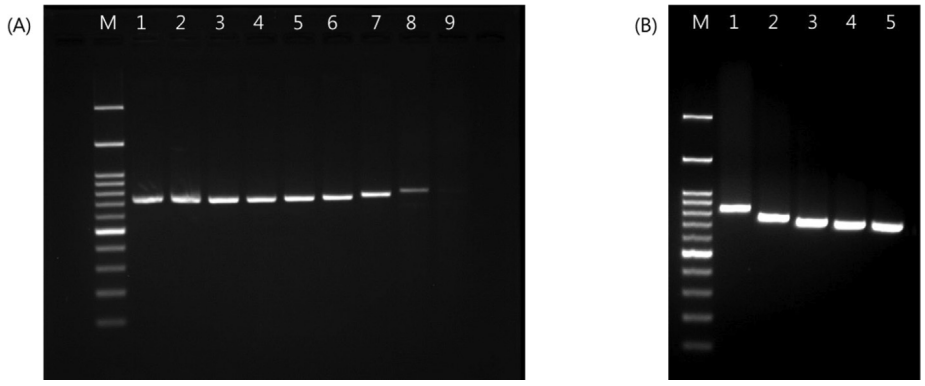

Fig. 2. Results of optimizing the COII PCR conditions to identify M. lusoria. (A) Determination of the optimal reaction temperature. Lane 1−9: PCR performed at 48 °C, 50 °C, 52 °C, 54 °C, 56 °C, 58 °C, 60 °C, 62 °C, and 64 °C, respectively. (B) Determination of the optimal number of reaction cycles. Lane 1−5: PCR performed for 26, 28, 30, 32, and 34 cycles, respectively. Lane M: 100 bp DNA ladder.

The detection limit of the PCR reaction was tested using two-fold serial dilutions of genomic DNA. The detection limit of the COII PCR was 2.5 ng−39.06 pg (Fig. 3). In previous studies, PCR analysis for the identification of clam species was performed using various DNA templates. For example, a 25 ng template was used for the razor clam species identification [13], a 2000 to 3000 ng template was used for carpet shell species [28], a 10 ng template was used for scallop species [12], and a 1 ng template was used for *Mytilus* species [17]. However, the PCR detection limit was not examined in these studies. Kang [29] developed a PCR method to differentiate tilefish species, and the primer limitation of a *Branchiostegus japonicus*-specific PCR was 0.01 ng/μL of DNA. Furthermore, in a separate study, the detection limit was between 0.002 ng and 0.0002 ng of DNA per reaction of real-time PCR for four commercially relevant crustaceans identification [30]. The results of this study suggested that the COII PCR has high sensitivity. Real-time PCR is an alternative method for improving the LOD. In order to verify the primer specificity, the DNA of several common bivalves in Taiwan were used for COII PCR. No specific amplicon was observed in *C. sinensis, C. fluminea, T. literatus, R. variegata*, or *M. mercenaria* (Fig. 4). The result showed good specificity of primer, and no cross-reaction was observed, potentially due to the low conservation of the COII gene among the various genera. This is conducive to the rapid identification of *Meretrix* species and to maintaining the specificity of the detection.

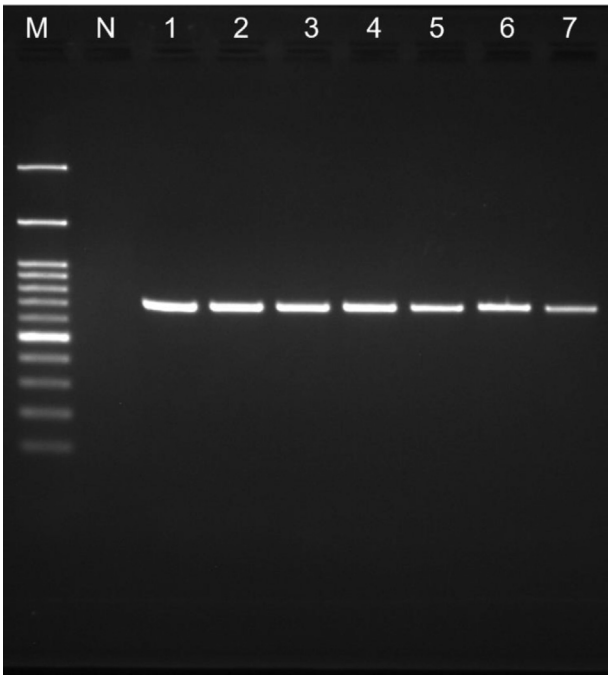

Fig. 3. LOD of the COII PCR primers. Lane N: negative control, lane 1−7: PCR performed using different concentrations genomic DNA of M. lusoria: 2.5 ng, 1.25 ng, 625 pg, 312.5 pg, 156.25 pg, 78.13 pg, and 39.06 pg, respectively. Lane M: 100 bp DNA ladder.

3.3. Comparison of the gel pattern of the COI, COII, and 18S PCR products

The PCR gel pattern of COII gene was applied for the rapid identification of four hard clam species, and compared with the common 18S and COI genes. The PCR products of these three genes were observed using agarose gel electrophoresis. The electrophoresis results of 18S rDNA suggested that the PCR amplicon lengths were highly similar for the four *Meretrix* species (see Fig. 5A). The COI results showed a larger fragment for *M. lamarckii*, while those of the other three species were similar (Fig. 5B). Therefore, the PCR gel pattern for the COI and 18S genes did not distinguish the four *Meretrix* species. However, the PCR gel pattern of COII gene

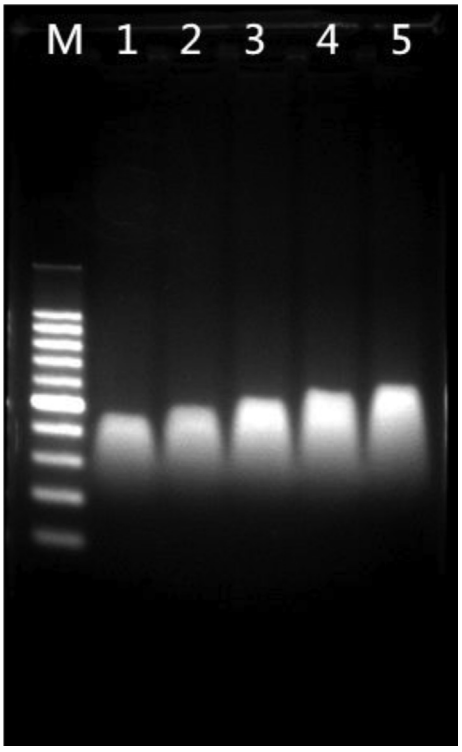

Fig. 4. Primer specificity of COII PCR detection. Lane 1: Cyclina sinensis, Lane 2: Corbicula fluminea), Lane 3: Mercenaria mercenaria, Lane 4: Ruditapes variegata, Lane 5: Tapes literatus. Lane M: 100 bp DNA ladder.

showed different fragment lengths for the four *Meretrix* clams (Fig. 5C). Lanes 1 to 4 indicate *M. lamarckii* (884 bp), *M. lyrata* (611 bp), *M. petechialis* (683 bp), and *M. lusoria* (722 bp), respectively. The differences in PCR amplicons were visible directly, and the gel pattern of the COII PCR could be used to distinguish *M. lusoria* from the other three hard clams. After DNA sequencing, the COII PCR amplicons were identified at the species level using BLAST; the query cover was 99%, and the identity was 97.91%−99.66% (Table 2). In eukaryotes, COI, COII, and COIII, which are encoded by mitochondrial DNA, form the catalytic core of the cytochrome c oxidase (COX). COX catalyzes the electron transfer from reduced cytochrome c to molecular oxygen in a process coupled to the transfer of protons across the inner membrane, thus contributing to the generation of the proton gradient that is used by ATP synthase to drive ATP synthesis [31]. In the literature, COII is seldom used as the main target gene for species identification or genetic relationship analysis. In this study, we found a region of high length variation in the COII of the four *Meretrix* clams. The variable region was located between the conserved regions, which were suitable for PCR primer design (Fig. 1). The amplicons were successfully amplified and directly observed using agarose gel electrophoresis. Four amplicon lengths representing four specific *Meretrix* species were identified. Our data indicate that COII is a potential DNA marker for identifying *M. lusoria* and three other bivalves (*M. lamarckii*, *M. lyrata* and *M. petechialis*).

3.4. Phylogenetic analysis

We reconstructed the phylogenetic relationships of four *Meretrix* species based on COI and COII genes' DNA sequences with *M. strigata* as the outgroup, using the maximum likelihood (ML) method (Fig. 6). The ML phylogenetic tree of COII gene

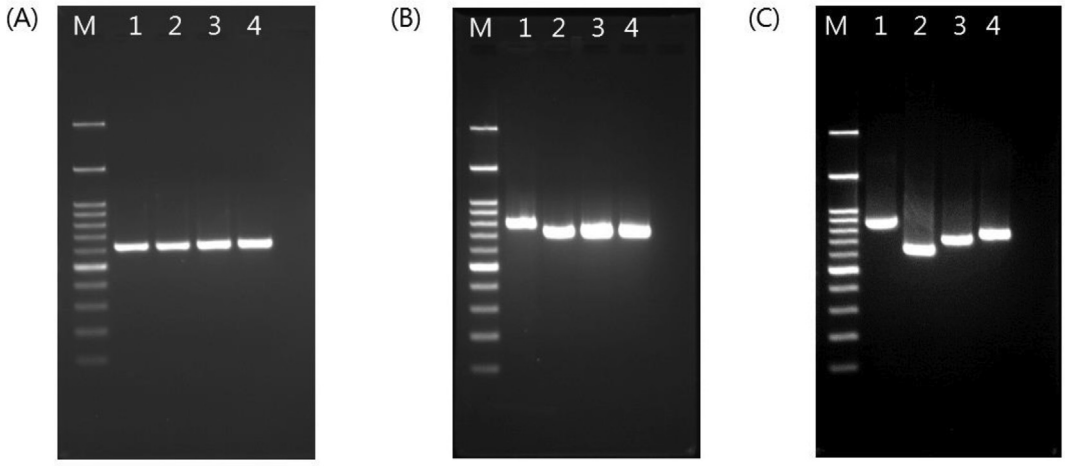

Fig. 5. Comparison of the agarose gel pattern of the 18S, COI, and COII PCR products. (A) Results of 18S rDNA primers. (B) Results of COI gene primers. (C) Results of COII gene primers. Lane 1: M. lamarckii, lane 2: M. lyrata, lane 3: M. petechialis and lane 4: M. lusoria. Lane M: 100 bp DNA ladder.

Table 2. Species identification results using the COII PCR products.

Source of *Meretrix* species	Length of COII PCR product	Accession of COII PCR product	Matched accession from BLAST	% Query Cover	% Identity	Matched species.
Taiwan	884 bp	ON921092	GU071281	99	98.75	*M. lamarckii*
Vietnam	611 bp	ON921093	KC832317	99	99.66	*M. lyrata*
China	683 bp	ON921095	EU145977	99	97.91	*M. petechialis*
Japan	722 bp	ON921094	MT418596	99	99.01	*M. lusoria*

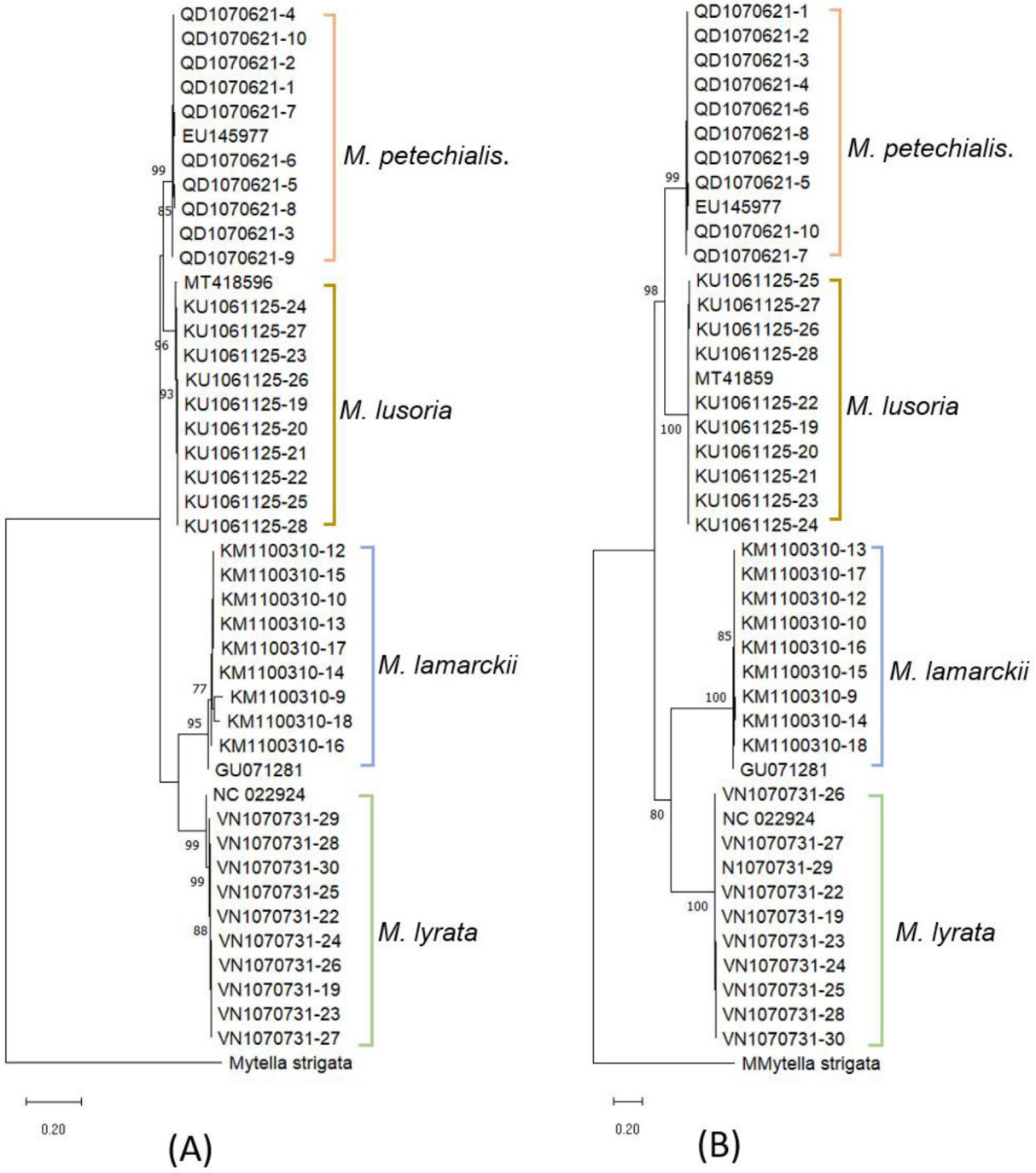

Fig. 6. The phylogenetic trees inferred from the nucleotide sequences of the COI (A) and COII (B) genes. The tree was reconstructed using the maximum likelihood (ML) method with MEGA v.11 based on the HKY + I model. Bootstrap values (500 replications) greater than 75% are shown at the branch nodes.

grouped the four *Meretrix* species into different clades with strong bootstrap support (Fig. 6B). The bivalves form Qingdao were grouped with *M. petechialis*, the bivalves from Kumamoto were grouped with *M. lusoria*, the bivalves from Kinmen were grouped with *M. lamarckii*, and the bivalves from Vietnam were grouped with *M. lyrata*. These results are consistent with those of the phylogenetic analysis of COI gene (Fig. 6A).

3.5. Verification of the COII PCR fragments by RFLP analysis

The amplicons length of the COII PCR could serve as a rapid approach to identify the four *Meretrix* species. Furthermore, the results of PCR also could be verified by RFLP analysis without DNA sequencing. A unique restriction site was chosen from COII gene sequences for four *Meretrix* species. According to the gel pattern of the RFLP, two fragments were observed for all *Meretrix* clams, but the lengths of the digested fragments were varied. For *M. lamarckii*, the 160 bp and 730 bp fragments were digested by *Pvu*II (Fig. 7A). For *M. lyrata*, the 460 bp and 150 bp fragments were digested by *Bgl*II (Fig. 7B). For *M. petechialis*, the 480 bp and 220 bp fragments were digested by *Eco*RV (Fig. 7C), and for *M. lusoria*, the 450 bp and 320 bp fragments were digested by *Bsp*HI (Fig. 7D). Furthermore, we also used the single *Hpy*188I to discriminate the four species. The restriction pattern of four *Meretrix* clams were differed after agarose gel electrophoresis (Fig. 8). The restriction fragments of *M. lamarckii* were about 550 bp and 420 bp. The restriction fragments of *M. lyrata* were about 320 bp, 250 bp and 120 bp. The restriction fragments of *M. petechialis* were about 550 bp and 350 bp. The restriction fragments of *M. lusoria* were about 480 bp and 150 bp. The agarose gel pattern of the RFLP fragments also successfully distinguished *M. lusoria* and the other three *Meretrix* clams. The species identification results of the PCR and RFLP fragments were

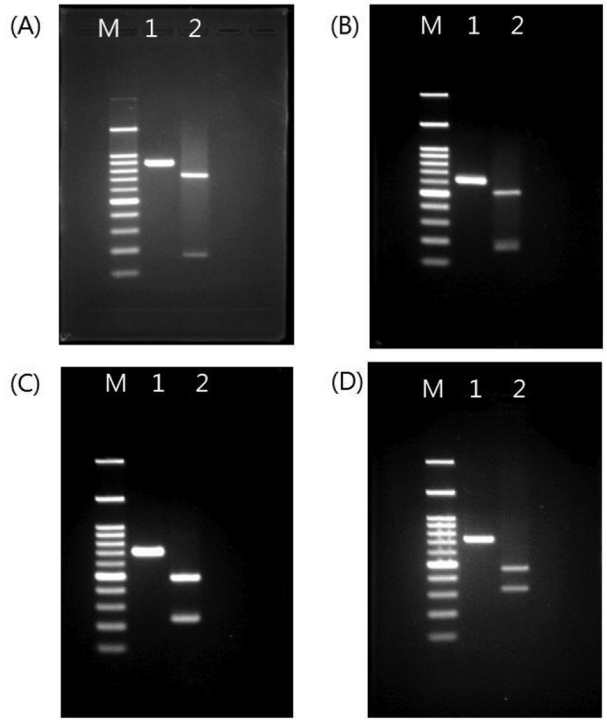

Fig. 7. Agarose gel electrophoresis of PvuII, BglII, EcoRV *and* BspHI *restriction enzyme digestions of the COII PCR products. (A) M. lamarckii. Lane 1: COII gene PCR amplicon, lane 2:* PvuII *restriction digestions of the COII gene PCR amplicon. (B) M. lyrata. Lane 1: COII gene PCR amplicon, lane 2:* BglII *restriction digestions of the COII gene PCR amplicon. (C) M. petechialis. Lane 1: COII gene PCR amplicon, lane 2:* EcoRV *restriction digestions of the COII gene PCR amplicon. (D) M. lusoria. Lane 1: COII gene PCR amplicon, lane 2:* BspHI *restriction digestions of the COII gene PCR amplicon. Lane M: 100 bp DNA ladder.*

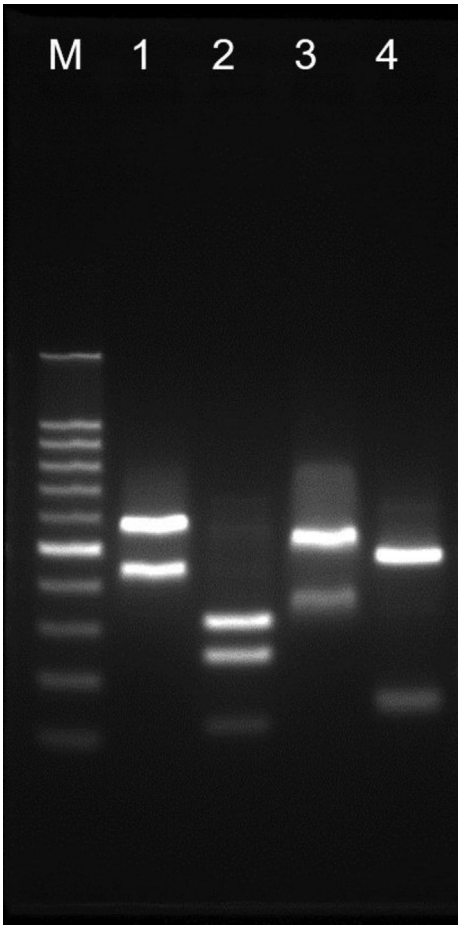

Fig. 8. Agarose gel electrophoresis of the Hpy*188I restriction digestions of the COII PCR products. Lane 1: M. lamarckii, lane 2: M. lyrata, lane 3: M. petechialis, and lane 4: M. lusoria. Lane M: 100 bp DNA ladder.*

identical when visualized using agarose gel electrophoresis. Species identification has been conducted for various bivalves RFLPs, in several target genes. Yamakawa & Imai [8] distinguished *M. lusoria* and *M. petechialis* using the COI PCR-RFLP method. Five razor clam species were distinguished by 5S rDNA PCR-RFLP [13]. Four scallop species were distinguished by ITS PCR-RFLP [12]. Three carpet shell clams were distinguished by alpha-actins [28] and ITS PCR-RFLP [11]. Freire [32] identified the razor clams *Ensis arcuatus* and *Ensis siliqua* by PCR-RFLP analysis of the ITS1 region. Oliveira Junior et al. [33] identified *Limnoperna fortunei* and *C. fluminea* by 18S rDNA PCR-RFLP. Moreover, a PCR-RFLP method that analyzes the PAP nuclear gene was used to identify mussel-based products (*Mytilus* spp.) [17]. Several novel DNA-based analysis methods were applied on bivalves or aquatic animals, such as genome-wide RAD-SNPs [34], next-generation sequencing (NGS) [35,36], and real-time PCR [18,30,37]. However, PCR and RFLP remain the most economic and convenient developed options.

4. Conclusion

In this study, we found a region of COII gene that varied appreciably in length in four *Meretix* clams and successfully used it to develop a PCR method rapidly identify *M. lusoria*, *M. petechialis*, *M. lamarckii* and *M. lyrata*. The limit of COII PCR detection was 39.06 pg and showed good specificity of primer. The lengths of the COII PCR amplicons were different and were observed using agarose gel electrophoresis. The gel patent was rapidly identified for *M. lusoria* and the three other *Meretrix* clams. The RFLP pattern also distinguished the four *Meretrix* species, and the RFLP results were consistent with the PCR results. The ML phylogenetic tree of the COII gene grouped the four *Meretrix* species into different clades with strong bootstrap support. These results are consistent with those of the phylogenetic analysis of COI gene. These results suggest that this COII PCR method can be used for routine and rapid species identification.

Conflict of interest

None of any author has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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