Identification of Four Mud Crab Species (Genus Scylla) based on High-resolution Melting (HRM) Analysis

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Short Report

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Abstract

In this study, we described the successful identification of four mud crab species (genus *Scylla*) by high-resolution melting curve analysis (HRM) of single nucleotide polymorphisms (SNPs). The complete mitochondrial genome sequences of *Scylla serrata*, *Scylla paramamosain*, *Scylla olivacea* and *Scylla tranquebarica* were aligned and screened for distinct fragments. A single pair of primers were designed to amplify the 203-bp consensus amplicon. As a result, a total of 96 samples from the four mud crab species were clearly separated. The melting profiles from HRM analysis were found to be distinct across the species tested, and a number of 28 SNP sites were confirmed by sequencing. This new identification method will be a useful tool for discrimination of the four *Scylla* species and contribute to the effective breeding management of these species.

Introduction

The mud crabs, *Scylla* spp. are a group of 4 commercially important portunid species. They are important components of the mangrove ecosystem and distributed widely throughout the Indo-West-Pacific region (Macnae 1968; Keenan et al., 1998). However, overfishing and the damage of natural habitat of mud crabs has been observed at varying levels in different countries as both national and international markets have developed (Lebata et al. 2009). In recent years, recovery efforts such as artificial breeding and release are in progress for the stock enhancement purpose (Le Vay et al. 2008; Lebata et al. 2009). Considering the distinct larval rearing procedure and population habitat of different *Scylla* species in artificial breeding and release program (Waiho et al. 2018), the accurate taxonomic assessment of genus *Scylla* is necessary for the conservation and recovery of local *Scylla* germplasm resources.

Because of their high morphological plasticity and the absence of distinct morphological diagnostic characters, it is difficult to identify *Scylla* species visually. In the previous studies, molecular identification methods based on the variation in product fragment length of mitochondrial DNA and nuclear ribosomal DNA have been established and facilitated the classification studies of *Scylla* (Imai et al. 2004; Ma et al. 2012). High resolution melting curve (HRM) is a relatively new technology in the field of DNA-based molecular diagnostics which has been proved to be an efficient, high-throughput genotyping method in the discrimination of various species (Morgan et al. 2011; Jin et al. 2015; Chen et al. 2018). The present study verified the mitochondrial DNA fragments that differed among the 4 *Scylla* species, and developed a useful and efficient molecular marker based on HRM to discriminate the 4 *Scylla* species.

Materials And Methods

Source of Samples and DNA Isolation

Samples of mud crab were collected respectively from Chilika of India (*S. serrata*); Sanmen of China (*S. paramamosain*); Kedah of Malaysia (*S. olivacea*), Kuala Ibai of Malaysia (*S. tranquebarica*). Genomic DNA of 24 individuals for each species were extracted from the muscle by standard proteinase-K
digestion and phenol-chloroform extraction. The quantity and purity of DNA samples were evaluated using NanoDrop 2000 (Thermo Scientific, USA).

**PCR amplification and HRM analysis**

The complete mitochondrion genome of the four species were downloaded from NCBI database (http://www.ncbi.nlm.nih.gov/) (accession numbers: FJ827761.1, FJ827758.1, FJ827760.1, FJ827759.1). Sequence alignment was conducted using ClustalX to examine the variation among species (Larkin et al. 2007). Primers were designed using the Primer Premier v. 5.0 program (PREMIER Biosoft International) for conserved flanking regions of the divergent sites. Design criteria included: primer pairs have a length of 20-35 bp and target amplicon size of 100-250 bp; annealing temperature between 50 and 60°C; the GC content of target fragments should be different from each other.

PCR amplification and HRM analysis were performed on a LightCycler®480 real-time PCR instrument (Roche Diagnostics) in a reaction mixture of 20 μL volume, containing 10 μL 1× LightCycler® 480 HRM Master Mix with ResoLight® dye (Roche Diagnostics), 10 pmol of each primer, 2.8μL MgCl₂, 100ng DNA, and adjusted with RNase-free water to a nal volume of 20 μL. The amplification conditions were detailed as follows: predenaturation at 95 °C for 5 min followed by 45 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s. After PCR amplification, products were denatured at 95 °C for 10 s, and HRM signals were collected from 60°C to 95°C at ramping increments of 0.2°C/s. LightCycler®480 Gene Scanning Software v. 1.5 (Roche Diagnostics) was used for data analysis. Genotypes were identified by examining normalized melting curves, difference and derivative plots of the melting data. All the samples were then sequenced in both directions on an ABI PRISM 3730 automatic sequencer to check the exact mutation type. Haplotypes of all the four species were identified using software DnaSP 6 (Rozas et al. 2017).

**Results And Discussion**

The accuracy of species identification is crucial for breeding, husbandry, and consumers. The overlapping of distribution areas and similarity of morphology make it difficult to identify *Scylla* species. HRM analysis is sensitive and can distinguish single-site mutations in a sequence, it has been widely used in biological diagnostics and species identification (Morgan et al. 2011; Jiang et al. 2014; Jin et al. 2015; Chen et al. 2018). Compared with existing gel-based species discrimination methods of *Scylla* species, HRM analysis is a very promising technique, which provides a rapid, sensitive and high-throughput alternative for species identification (Jin et al. 2015). In this study, a 203-bp fragment of mitochondrion sequence of the four mud crab species was amplified using the CSN9 primers 5'-AATTAACCTATGACATCATCTCATGG-3' (forward) and 5'- CGAGTTACATCTCGTCAT CATTG − 3' (reverse). The melting profiles from HRM analysis were successfully obtained and found to be distinct across the four mud crab species (Fig. 1). Tₘ calling analysis showed that the melting points of *S. serrata* and *S. paramamosain* were 79.55 and 80.12°C, while the *S. tranquebarica* and *S. olivacea* exhibited two melting
peaks at 78.12 and 82.04, 78.42 and 84.40°C respectively. These results proved that HRM is a sensitive technique for distinguishing these four species.

Sequencing analysis was performed in order to confirm the species-specific mutation which resulted in the distinct HRM profiles. A number of 9 haplotypes were identified across all the sequenced samples, including 2 for *S. serrata*, 3 for *S. olivacea*, 2 for *S. paramamosain*, and 2 for *S. tranquebarica* respectively. The alignment of haplotype fragments was presented in Fig. 2. The results demonstrated that the distinct HRM curves of each *Scylla* species were due to the interspecific differences in a number of SNPs. In total, 28 SNP sites were detected from the 203-bp amplicon of the target region. Transitions were observed at twenty-seven sites and transversions at one site. These sequencing results proved the robustness of HRM analysis to distinguish the four *Scylla* species with only one pair of primers.

In conclusion, the results obtained in this study indicated that the consensus 203-bp fragment of mitochondrial DNA sequence contained SNPs was appropriate for HRM analysis, the distinct melting profiles of the four mud crab species provide a rapid and high-throughput method for the discrimination of *Scylla* species, which will be a useful tool for the genetic research and establishment of effective breeding management of these species.

**Declarations**

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**Conflicts of interest/Competing interests:**

The authors declare that there are no conflicts of interest regarding the publication of this paper.

**Ethics approval:**
The methods used in the study were ethically approved by Ningbo University, China.

**Availability of data and material:**

The data used to support the findings of this study are included within the article.

**Code availability:**

Not applicable.

**Authors' contributions:**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yongliang Li, Ronghua Li, Xiayue Chen, Chunlin Wang, Changkao Mu, Weiwei Song. The first draft of the manuscript was written by Yongliang Li and Ronghua Li, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

Discriminations of single-nucleotide polymorphisms (SNPs) among four genus Scylla species using high-resolution melting analysis A Normalized and temp-shifted difference plot. B Normalized melting curves. C Melting peaks. (a) S.serrata, (b) S.paramamosain, (c) S.tranquebarica, (d) S.olivacea
Figure 2

Alignment of haplotype fragments from the mitochondrial DNA sequence of the four species using the pair of primers. Nucleotides identical to the sequence on the top are shown with dots.