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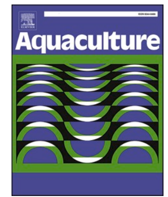

Short communication

High resolution melting analysis: A rapid and sensitive method for detection of mutations induced by CRISPR/Cas9-based genome editing in oysters

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ABSTRACT

Genome editing using CRISPR/Cas9 methodology holds much potential to accelerate the genetic improvement in aquaculture, and has been extensively tested and applied to various aquaculture species. However, screening and identifying successfully edited individuals based on sensitive, low-cost, and rapid methods remains challenging. Here, we developed and optimized a high-resolution melting analysis (HRM) assay to screen CRISPR/Cas9-edited larval oysters. The target amplification size range was optimized to 100–200 bp. Compared with fluorescent dyes SYBR Green and SYTO-9, saturated dye EvaGreen showed high stability and repeatability when used in PCR master mixes. The HRM assay was sensitive enough to detect a temperature shift when a mixture of 10% mutant colonies and 90% WT colonies were present. Sanger sequencing analysis confirmed that oysters with indel mutations could be successfully identified by the HRM method. Together, these data indicate that the HRM assay developed in this study is a reproducible, sensitive, and efficient method for rapidly screening and identifying gene-edited oysters.

1. Introduction

The CRISPR/Cas9 technology is a revolutionary breakthrough in genome editing and has been widely utilized to induce targeted mutations to investigate gene function (Yang et al., 2022). In recent years, with the rapid application of CRISPR/Cas9 for gene editing in more than 20 aquaculture species, the technology is widely applied to the discovery of gene function in fish, shrimp, mollusks and other species (Chan et al., 2022; Li et al., 2021a;Li et al., 2021b;Sun et al., 2020; Wang, 2022; Zhang et al., 2018). Therefore, adopting a fast and efficient screening method to identify mutants in gene-edited populations is crucial. In oysters, Yu et al. (2019) investigated mutation efficiency by incubating purified DNA fragments with sgRNA and Cas9 in vitro and analyzing them with the agarose gel. The method is time-consuming and unable to accurately predict the outcome in vivo. In addition, several other methods for gene editing efficiency assessment have been reported in mollusks. In marine gastropods, the mCherry reporter was integrated into *β-Catenin* to measure the efficiency of the CRISPR/Cas9 editing system. The approach is inappropriate for standard assays that directly confirm gene function (Perry and Henry, 2015). In the case of *calaxin* gene editing in *Lottia goshimai*, the gene was featured by very short exons and extensive sequence variation in introns, which made it challenging to measure the rate of mutant sequences with a conventional T7E1 assay or direct sequencing of PCR products (Huan et al., 2021).

In the past few years, several other screening methods were developed to assess the efficiency of CRISPR/Cas9 induced, such as double TaqMan probes real-time PCR method (Mock et al., 2016), PAGE-based genotyping assay (Ramlee et al., 2015), and annealing at critical temperature PCR (ACT-PCR) analysis-based assay (Hua et al., 2017). These methods, however, suffer from limitations, such as requiring special enzyme cutting sites, and being time-consuming, and labor-intensive (Vossen et al., 2009). Additionally, Abe and Kuroda (2019) successfully produced gene-edited snail *Lymnaea stagnalis* at the G2 generations with homozygous genotypes (Abe and Kuroda, 2019), but most mollusks, including oysters, only produced genotypically modified mosaic F0 generations via CRISPR/Cas9 injection (Li et al., 2021).

High-resolution melting curve (HRM) analysis is a powerful technique for mutation screening based on fluorescence quantitative PCR (Ririe et al., 1997). This technique utilizes fluorescent dyes, such as SYBRGreen I, LCGreen, SYTO-9, and EvaGreen, which bind to double-

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stranded DNA (dsDNA) and generate a change in fluorescence signal during melting in real-time (Ebili et al., 2017). The outcome of HRM analysis is mainly influenced by three factors: the length of the DNA sequence fragments, the GC content, and the degree of complementarity between the strands (Druml and Cichna-Markl, 2014). HRM has been widely applied to screen unknown gene mutation (Bilbao-Sieyro et al., 2014), SNP-typing (Liew et al., 2004), DNA methylation (Farmen et al., 2014), and other studies (Vossen et al., 2009; Samarut et al., 2016; Ge et al., 2015; Guo et al., 2011). Furthermore, HRM is made available to detect dose-dependent mutation induction, which is difficult to measure in somatic samples (Raposo et al., 2017). Meanwhile, HRM can be employed to diagnose TALENs induced mutation in zebrafish at multiple loci (Dahlem et al., 2012). Thomas et al. (2014) utilized HRM maps to reveal genotype-phenotype associations between mutant embryos from F0 intercrosses and screened mosaicism in F0 zebrafish as low as 5%. Hence, the real-time fluorescent quantitative PCR instrument with accurate temperature control can assay single-base variants, small insertions or deletions caused by gene-editing (Li et al., 2018).

In this study, we demonstrate the application of HRM analysis in screening gene mutations in Pacific oyster (*Crassostrea gigas*) larvae following microinjection of CRISPR-Cas9 complexes system into fertilized eggs. Our approach provides a simple and flexible operation with high sensitivity and specificity for the assay of heterozygous alleles. These findings suggest that HRM is a promising tool for high-throughput mutation assays in the F0 generation of gene-edited oysters.

2. Materials and methods

2.1. Preparation of mutant oyster larvae and DNA

Two candidate genes, *CgTYR-2* (LOC105344040) and *CgTYR* (LOC105329903) were selected as targets for gene editing in *C. gigas*. Two gRNAs were designed using the online tool CRISPOR (http://crispor.tefor.net/) (Table 1). Firstly, we design a 500 bp amplicon with specific primers near the target sites to avoid the presence of the SNPs indirectly affecting the judgment of HRM (Table 2). Then, Cas9mRNA and gene-specific sgRNAs were generated as previously described (Li et al., 2021c), and delivered into one cell stage oyster embryos by microinjection. The injected embryos were cultured in filtered seawater at 22 °C for 24 h, and then the gDNA was extracted from D-shaped larva using Chelex®-100 method (Li and Kijima, 2005). Simultaneously, to obtain homozygous mutant DNA, the DNA fragment amplicons of parts of mutant gDNA about 500 bp containing the target sites were performed by PCR with 2× Taq Plus Master Mix II (Vazyme, China), and specific primers (Table 2). The PCR fragments of 500 bp were sequenced by Sanger sequence and then the fragments with target site mutations were purified (Sangon Biotech). The target fragment was cloned into pMD19-T vector (Takara, Japan) and single clones were randomly selected for DNA sequencing with M13F primer. At least three different mutant genotypes of five clones were induced to complete the following experiments for each gene.

2.2. Mosaic degree of simulated oyster larvae mutation

To evaluate the limit of detection (LOD) by HRM, a series of gradient diluted plasmid DNAs were prepared by mixing plasmid homozygous mutation and wild types (WT) plasmid DNA. Prior to mixing, the concentrations and quality of the plasmid DNAs were evaluated using the NanoDrop 2000 UV/vis spectrophotometer. Finally, the samples were mixed according to the proportion of homozygous mutant plasmid/WT plasmid at the ratios of 100%, 75%, 50%, 25%, 10%, 5%, 1%, and 0%.

2.3. High-resolution melting analysis

The primer sequences and amplification sizes used for HRM analysis were shown in Table 1. Three fluorescent dyes were selected for the experiments. The PCR reaction system (20 μL) of SYTO-9 Dye consisted of 2 μL of sample DNA (at least 5 ng/μL), 0.5 μL of each primer (10 μM), 2.5 μL of dNTPs mixture (2 mM each), 2.5 μL 10× buffer for Taq DNA Polymerase, 1 μL fluorescent dyes (2 μM SYTO-9, Thermo, USA), 0.25 μL rTaq DNA polymerase (Takara, Japan), and 10.75 μL double distilled water. The other two dyes, ChamQ SYBR Color qPCR Master Mix (Vazyme, China) and EvaGreen® qPCR Master Mix (ABI, USA), were also carried out according to the manufacturer's protocols.

The amplification was generated by the touchdown PCR protocol on the LightCycler®480 real-time PCR system (Roche, Switzerland): first denaturation at 95 °C for 5 min, then 55 cycles of denaturation at 95 °C for 40 s, annealing and extension for 40 s at 62 °C for the first cycle and thereafter at 0.5 °C decrease each for 10 cycles, and a final extension at 72 °C for 40 s. Following amplification, the products were denatured at 95 °C for 1 min, and then annealed at 40 °C for 1 min to form DNA duplexes randomly. Melting curves were generated by heating samples from 60 to 90 °C with 25 data acquisitions per degree. All samples were run in duplicate, and a negative control of RNase-free water was induced during each run of the PCR to check for contamination.

2.4. Data analysis

Fluorescence was monitored during the entire heating process, and genotypes were determined by examining the normalized melting curve, temperature-shifted melting curves, and the melting-peaks display with the GeneScanning Analysis module of the LightCycler®480 Software (Roche) (Vaughn and Elenitoba-Johnson, 2004). The GeneScanning module plotted the negative first derivative of sample fluorescence against temperature to produce a melting curve. To estimate the accuracy of this method, all the amplicons obtained from HRM analyses were subjected to Sanger sequencing (BGI Biotech). The obtained sequences were then aligned by utilizing the ClustalW (Lynnon Biosoft) software to verify their accuracy and consistency.

3. Results

3.1. Optimization of key HRM parameters

To establish a HRM method with high sensitivity and accuracy, we used plasmid DNAs from bacterial colonies containing *CgTYR-2* homozygous mutant as the template to evaluate the effects of amplification sizes and fluorescent dyes on the assay (Fig. 1). Five pairs of *CgTYR-2* gene primers were designed (Table 1), which produced amplicons of varying sizes of 84 bp, 117 bp, 128 bp, 196 bp and 238 bp, respectively. The resulting amplicons were analyzed using HRM, which generated unique melt curves for different fragment sizes (Fig. 1). The data showed

Table 1
Primers used in sgRNAs synthesis.

Primer name	Sequnce (5′ - 3′)	Application
CgTYR-2-sgRNA-F	GATCACTAATACGACTCACTATA***GGTGCGGGCTCGGTGATTGG***GTTTTAGAGCTAGAAAT	
CgTYR-sgRNA-F	GATCACTAATACGACTCACTATA**GGGGGCAACCGTCCAATACA**GTTTTAGAGCTAGAAAT	sgRNA synthesis
SgRNA-R	AAAAGCACCGACTCGGTGCC	

Note:The bold sequences in *CgTyr-2*-sgRNAs and *CgTYR*-sgRNAs are the gene-specific target sequences within the respective sgRNAs. The underlined GG in sgRNAs were added by PCR primer for correct T7 transcription.

Table 2
Primers used in PCR, HRM and sequencing analysis.

Primer name	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)	Length (bp)	Application
TYR-2-HRM-1F/1R	TGTTATCATTTGATCCCTCCTATTTA	CGAGATGATGGACCCCACCACCTCCA	84	HRM assay
TYR-2-HRM-2F/2R	GACCGATCTCTGCACAATGGATATATT	CGAGATGATGGACCCCACCACCTCCA	117	
TYR-2-HRM-3F/3R	TGTTATCATTTGATCCCTCCTATTTA	CTGACTGGACCCTTTGGAAGAT	128	
TYR-2-HRM-4F/4R	TGTTATCATTTGATCCCTCCTATTTA	GGTTCCAGTGGGGGTTCTGAAT	196	
TYR-2-HRM-5F/5R	GGATATATTATAAAGGACGACA	GGTTCCAGTGGGGGTTCTGAAT	238	
TYR-HRM-F/R	GAAACTTTGCCGCCAAAAACG	GAAACTTTGCCGCCAAAAACG	89	
TYR-2-F/R	GGTTTATCTCCTGGTGTAAGTCT	ATCTTCCAAAGGGTCCAGTCAG	492	Mutation detection
TYR-F/R	TGATAGAAAACCTGGAATACCC	GTTGTCGATGAGGTTTAACAAT	413	

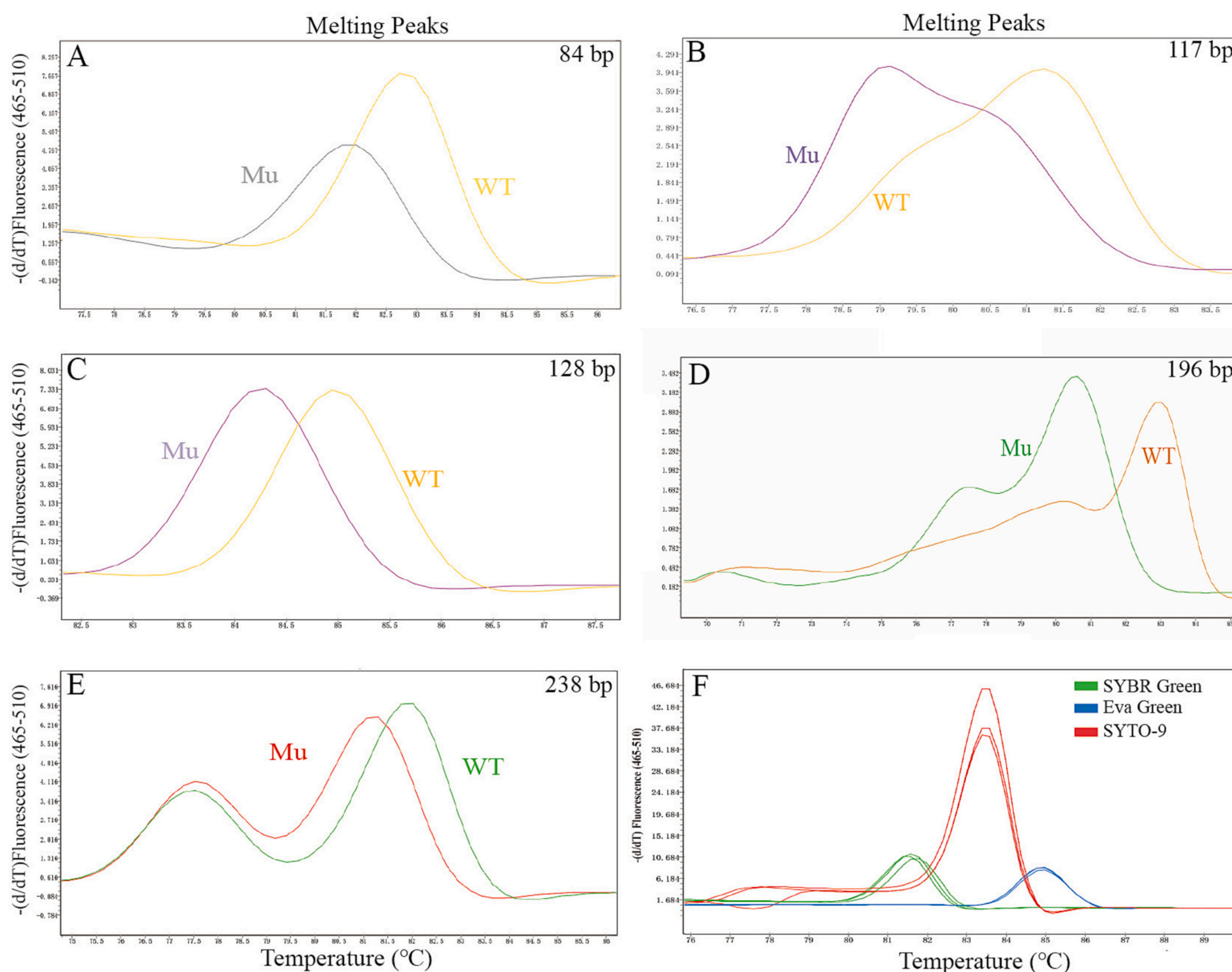

Fig. 1. HRM optimization on amplicon size and fluorescence dyes. (A - E) Normalized melting peaks of the HRM assays with varied primer sets, such as 84 bp, 117 bp, 128 bp, 196 bp, and 238 bp. Mutation: DNA from bacterial clones of homozygous mutations; WT: DNA of wild-type clones. (F) Normalized melting peaks of the HRM assays employing primers *TYR-2*-HRM-3F/3R with three different fluorescence dyes: SYBR Green I (green), Eva Green (bule), and SYTO-9 (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that when the amplicons were too long, the melting profiles at a given locus contained two or more peaks, which made the analysis more complex and challenging (Fig. 1D and E). A clear shift in the unique peak between the melt curves of *CgTYR-2* mutant and WT samples were observed, especially when using the *CgTYR-2*-HRM-3F/3R primer pair that gave a 128 bp amplicons (Fig. 1C). This finding indicates that the melting temperature (Tm) of the mutant and WT amplicons differed significantly, which allowed for accurate distinction between the two genotypes using HRM analysis.

Based on the results of amplicon size optimization, primer pair *CgTYR-2*-HRM-3F/3R was selected to optimize fluorescent dye selection. We evaluated the performance of three frequently used fluorescence dyes, namely SYBR Green I, EvaGreen Mix, and SYTO-9. According to the fluorescence intensity of the samples, we found that SYTO-9 exhibited the highest fluorescence signal intensity, whereas the EvaGreen Mix group showed the most stable and reproducible fluorescence signal (Fig. 1, F). These results indicated that the EvaGreen Mix fluorescent dye and amplicons of around 100 bp were the optimal

conditions for performing HRM assays, as they provided a good balance between stability of fluorescence signal and reproducibility of Tm values.

3.2. Limit of detection of the HRM assay

The efficiency of obtaining mutant individuals through CRISPR/Cas9 mediated gene editing can vary dramatically across different organisms and experimental conditions. Furthermore, in the F0 generation of gene-edited organisms, the presence of mosaicism is a common occurrence. Therefore, it makes sense to identify positive signals from gene-edited individuals by applying sufficiently sensitive screening methods. To determine the sensitivity of the technique, we prepared templates by mixing plasmid DNA of homozygous mutant and WT plasmid DNA at different ratios of 100%, 75%, 55%, 25%, 10%, 5%, 1% and 0%, respectively. Subsequently, the LOD were evaluated for the HRM assay. Although qPCR showed strong fluorescence signals and similar values of periodic threshold (Ct) for all samples of mutant plasmid DNA at different concentrations, HRM analysis revealed different melt curves and normalized melt curves across different concentrations (Fig. 2). Notably, as the concentration of the mutant DNA decreased, the difference curve's Δfluorescence also decreased (Fig. 2). The highest Δfluorescence values were observed in the sample with 100% mutant DNA, while the lowest Δfluorescence was obtained in samples containing at least 10% mutant DNA. Δfluorescence was undetectable in samples with 5% and 1% mutant DNA and 100% WT DNA. The HRM technique demonstrated high reproducibility with the absence of false positives or negatives. The limit of detection was achieved when detecting a temperature shift in a mixture of 10% mutant DNA and 90% WT DNA (Fig. 2).

3.3. Feasibility of detection of the HRM assay

To further explore the sensitivity and efficiency of HRM assay, six different mutant genotypes were selected from the ten plasmids of two genes identified by sequencing (Fig. 3A and D). Based on the results of optimization experiments, primers Cg*TYR-2*-HRM-3F/3R and *CgTYR*-HRM-F/R and the EvaGreen Mix fluorescent dye were selected to further optimization of the HRM detection. The conventional PCR results demonstrated that each primer pair could amplify a particular DNA fragment of the predicted size, but without variation between WT and mutants (data not shown). On the contrary, melting peaks with Tm value shifting could be observed among the samples in the HRM analysis (Fig. 3B and E). After normalization, different genotypes could be readily distinguished. In the normalized melt curves, the five *CgTYR-2* samples form three distinct groups: group 1 (A1 and A4), group 2 (A3 and A5), and group 3 (A2) (Fig. 3B). The sequencing results showed that samples A3 and A5 were homozygous for a 3 bp insertion (ATT) (Fig. 3A). The sequence variations were undetected in sample A2, which served as the WT control. Samples A1 and A4 showed either large fragments deletion or insertions. Similarly, the HRM analysis was carried out on the five *CgTYR* samples, and gave the following results: both B3 and B5 displayed identical melting peaks (Fig. 3E). The sequencing and HRM results were in agreement, indicating that the 10 samples were accurately identified by the HRM assay. In particular, sample A3 displayed a homogeneous two-base pair (TA) deletion, whereas sample A6 exhibited a homogeneous single-base pair insertion.

3.4. Screening and genotyping analysis of gene-edited TYR-2 and TYR oysters

We targeted two sites, *CgTYR-2* and *CgTYR*, for gene editing through microinjection of CRISPR-Cas9 complexes, and the gDNA was extracted six injected D-shape larvae. The gDNA samples were then analyzed by performing HRM with *CgTYR-2*-HRM-3F/3R and *CgTYR*-HRM-F/R primers to detect mutations at the respective target sites (Fig. 4A and B). To further confirm the HRM genotyping analysis results, all amplified DNA fragments were purified and analyzed by DNA sequencing (Fig. 4C). Samples with a melting curve differing from that of the WT were indeed identified as bearing mutations through sequencing of the HRM PCR products. In contrast, sequence alterations were not observed in the samples with WT or borderline melting profiles. As a closed-tube experiment, our method allowed the addition of more fluorescent dyes without affecting DNA integrity compared to conventional PCR. This

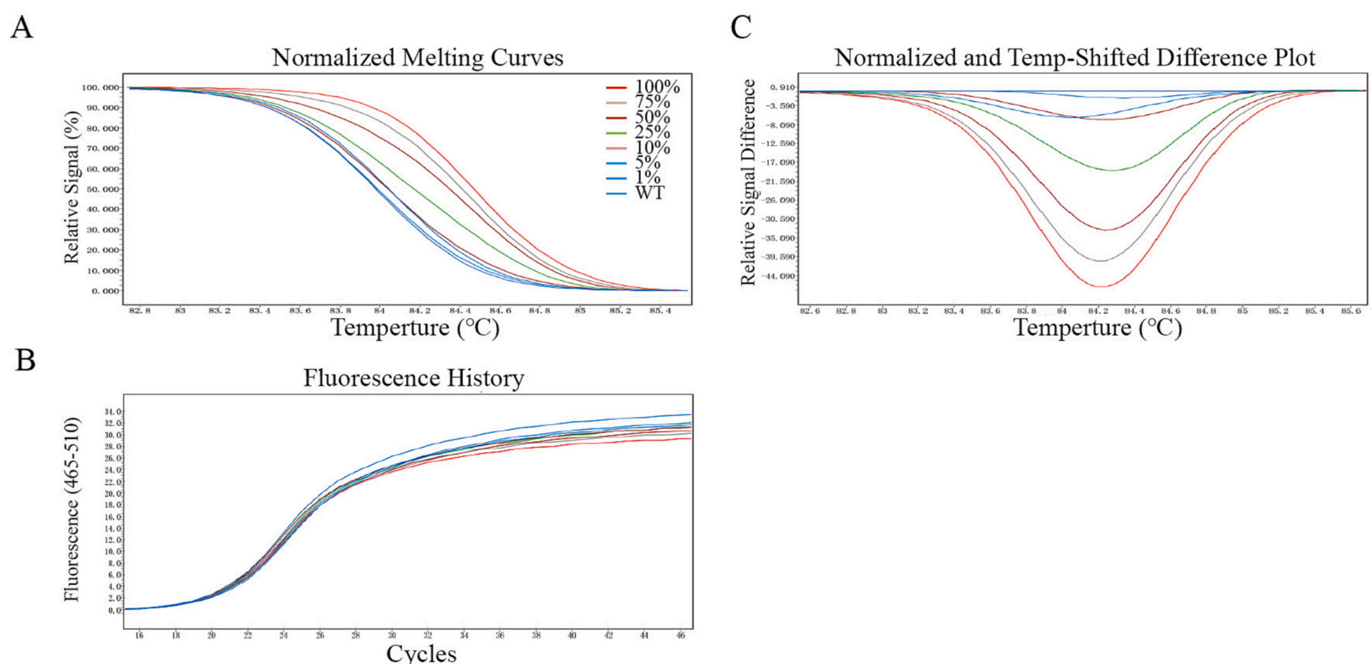

Fig. 2. Evaluation of the sensitivity of HRM assay.
Plasmid DNA of the homozygous mutant was mixed with WT plasmid DNA at a ratio of 100%, 75%, 50%, 25%, 10%, 5%, 1%, and 0%. (A) The normalized high-resolution melting curves; (B) The qPCR amplification curves; (C) The normalized and Tem-Shifted difference plots.

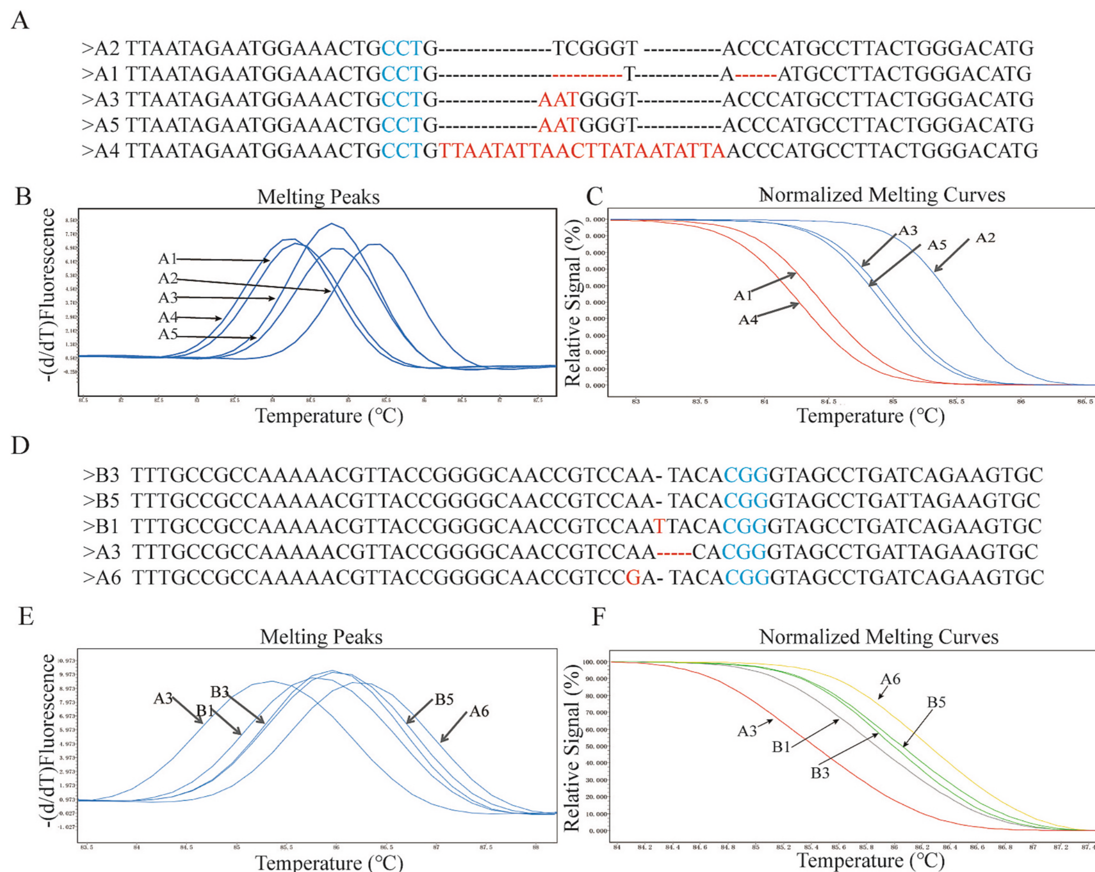

Fig. 3. The feasibility of genotyping by HRM.
DNA plasmids from clones of TYR-2 (A - C) and TYR (D - F) mutants were utilized as DNA templates for HRM and Sanger sequencing analyses. (A) Sanger sequencing results of the diverse types of mutants (A1-A5). (B) The melt curves of HRM analysis of 5 samples (A1-A5); (C) The normalized high-resolution melting curves of 5 samples (A1-A5); (D) Sanger sequencing results of the homozygous samples (A3, A6, B1, B3, B6). (E) The melt curves of HRM analysis of 5 samples (A3, A6, B1, B3, B6); (F) The normalized high-resolution melting curves of 5 samples (A3, A6, B1, B3, B6).

feature held practical implications as it obviated the need for sequencing confirmation and substantially reduced the cost and workload of mutant screening.

4. Discussion

With the complete oyster genome sequence available (Peñaloza et al., 2021; Zhang et al., 2012), the reverse genetic techniques by gene knockout and knockin provide crucial insights into gene function. These techniques, when applied in genome-wide studies, enable large-scale genotypic and phenotypic analysis and are of significance in aquaculture research. With the advent of CRISPR/Cas9 and TALEN, these techniques are now easily accessible and highly proficient for use in oysters (Jin et al., 2021; Li et al., 2021a; Yu et al., 2019). The HRM assay we developed here enables high-throughput calculation of mutation rates through, thereby reducing the number of injected embryos that need to be analyzed by DNA sequencing, which was a significant limitation in previous methods of screening (Montgomery et al., 2007; Rojo et al., 2020). In this study, we demonstrated that HRM is an extremely flexible method that can be applied across a wide range of contexts, as long as PCR amplification can be obtained covering the target site. Importantly, HRM can be performed in both 96 and 384 well plates with a closed-tube reaction, making it an ideal high-throughput genotyping assay (Simko, 2016). And the HRM is exceptionally sensitive when embryonic mutations result in the formation of unstable heterozygous double strands (Fuster et al., 2009; Wang et al., 2015). Our studies further confirmed that HRM was reliable for screening mutations in CRISPR/Cas9 edited embryonic oysters.

It is crucial to optimize primers and dyes to ensure unbiased amplification of each allele in the sample during the HRM assay. To achieve accurate genotyping, we recommend applying online tools for primer design and melting curve prediction (Dwight et al., 2011). Additionally, in heteroduplexes with mismatches probe-alleles, the melting temperature was usually lower compared to those with perfectly matched probe-target DNA, and heterozygous samples displayed two distinct peaks (Samarut et al., 2016). To prevent errors caused by heterozygous mutant genotypes during primers and dyes selection, homozygous mutants and WT DNA samples were utilized. In general, smaller amplicons yield a larger difference in the melting temperatures. It has been demonstrated that the ideal size of amplicon for HRM in oysters is around 100 bp. However, the detection of larger differential spots were limited by size of amplicon. Amplicons that were too long (more than 200 bp) tended to result in double peaks in the melting curve analysis, which complicated the data analysis. Furthermore, Huan et al. (2021) suggested that genetic polymorphisms should be carefully considered when CRISPR-based gene editing was used in mollusks. To minimize this effect, we suggest designing a 200 base-pair amplicon near the target when selecting a target for gene editing to avoid the presence of the SNPs indirectly affecting the experimental results.

Another consideration of the HRM method is that the choice of dyes because of their potential effect on the melting curves (Wittwer, 2009). HRM assays developed using saturated mix dyes provided several advantages for identifying CRISPR/Cas9-edited mutant oysters, including high sensitivity, high resolution, and simple operation. Additionally, analysis of melting curves can extend the dynamic range of initial template quantification when amplification is monitored with double-

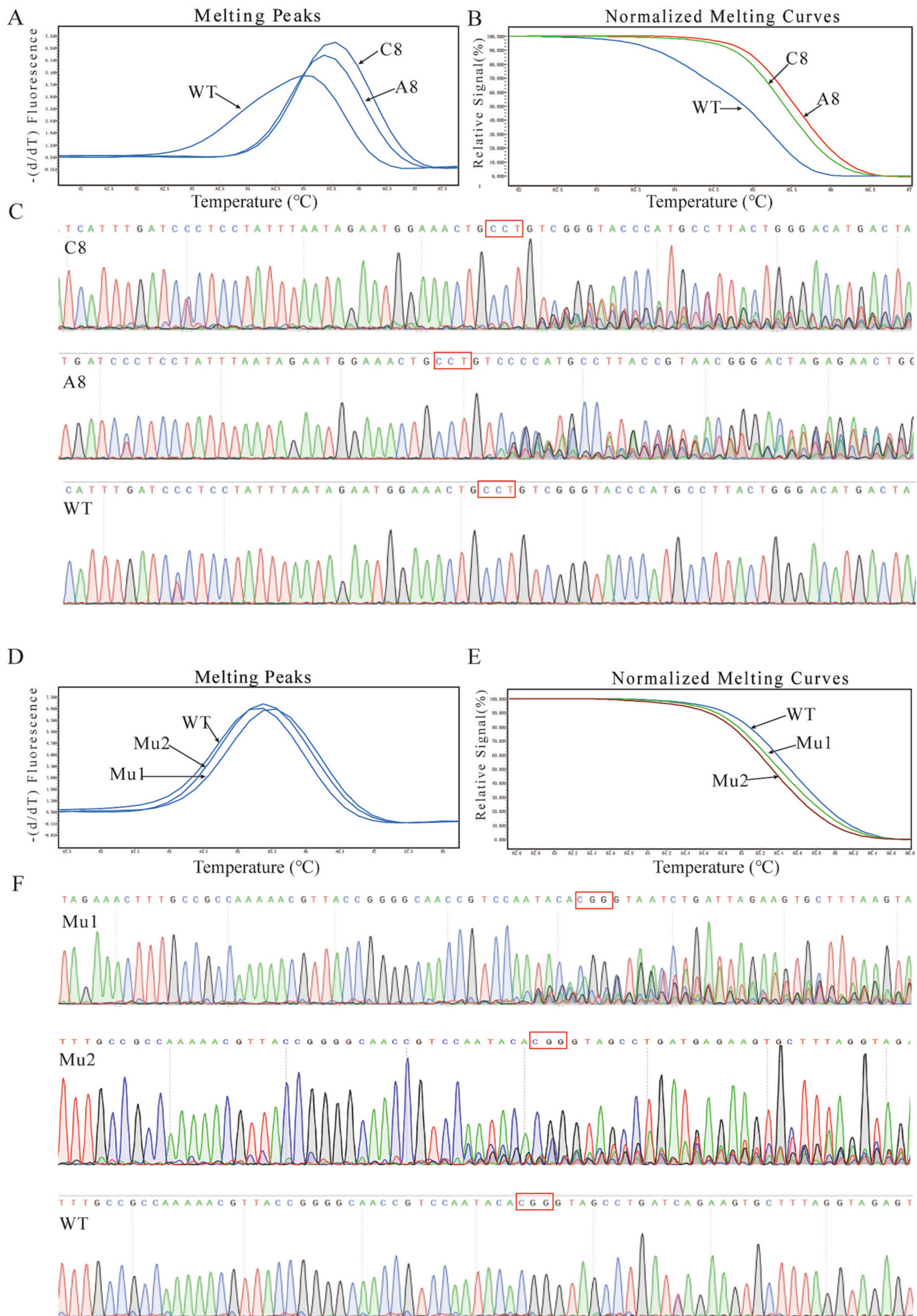

Fig. 4. Genotyping analysis of gene-edited larval oysters using HRM.
(A-B): The normalized high-resolution melting curves of 3 *CgTYR-2* gene-edited oysters (WT, C8 and A8). (C): Sanger sequencing results of WT-*CgTYR-2* and *CgTYR-2* mutant oysters (C8, A8). (D-E): The normalized high-resolution melting curves of 3 *CgTYR* gene-edited oysters (WT, Mu1, Mu2). (F): Sanger sequencing results of WT-*CgTYR* and *CgTYR* mutant oysters (Mu 1 and Mu 2).

stranded DNA specific dyes. In contrast to SYBR Green, high concentrations saturation dyes (e.g., EvaGreen and SYTO-9) provided no effect on the activity of DNA polymerase, making it possible to label the PCR product along its entire length and therefore screen all melting domains (Thomas et al., 2014; Vossen et al., 2009). Here, the EvaGreen Mix was applied on account of its reproducibility. In addition, the shape and position of the DNA melting curve were determined by the GC/AT ratio, length, and sequence of the amplified product. Therefore, it is crucial to design primers that avoid non-specific amplification (Bilbao-Sieyro et al., 2014). Moreover, before carrying out melting curve analysis, the template must be amplified in the presence of a dsDNA binding dye (Ririe et al., 1997).

Furthermore, since the gene editing process is accompanied by cell division of fertilized eggs, which will result in mosaicism in the F0 generation, leading to the genetic heterogeneity in the F0 population (Yen et al., 2014). In this study, HRM can be applied to detect samples with the same heterozygous background, which is a mixture of 10% mutant DNA and 90% wild-type DNA. Additionally, as gene editing mutations occur randomly in F0 oysters and mutation types are unpredictable, HRM can be employed to detect gene-edited *CgTYR* and *CgTyr-2* in oyster larvae. Therefore, the method is broadly applicable for verifying gene editing in oysters. However, to confirm the genotype, sequencing is necessary to distinguish between heterozygous and homozygous mutations, although HRM remains a reliable preliminary high-throughput screening method.

To summarize, HRM is an effective, fast, sensitive, and high-throughput screening method for identifying CRISPR/Cas9-induced mutant oysters. The method will facilitate the screening of individuals in germplasm repositories for potential sequence variants in target genes, reduce the number of sequencing steps required, and improve the cost-effectiveness of allele mining. Furthermore, running in a closed-tube system allows for more accurate assay results and does not interfere with subsequent analyses, such as cloning of mutant genotypes or direct sequencing for quantification of fragment analysis. This allows for precise sample selection with mutation characteristics.

CRediT authorship contribution statement

Qian Li: Investigation, Conceptualization, Formal analysis, Writing – original draft. **Hong Yu:** Investigation, Supervision, Writing – review & editing. **Qi Li:** Supervision, Conceptualization, Resources.

Declaration of Competing Interest

There are no conflicts to all authors for this paper.

Data availability

Data will be made available on request.

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