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An effective method for parentage determination of the clam (*Meretrix meretrix*) based on SSR and COI markers

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ABSTRACT

Because of the high frequencies of null alleles at microsatellite loci in mollusks, it is difficult to enhance the accuracy of parentage assignment in closely related families by increasing the number of loci. To develop an effective approach, microsatellite markers combined with mitochondrion COI marker were used for parentage assignment with computer simulations and experimental verification in the clam, *Meretrix meretrix*. In the present study, simulations based on allele frequency data from the candidate parents (sample II) demonstrated that combined exclusion probability of the five microsatellite loci (0.991 for Excl 1 and 0.995 for Excl 2) were not much lower than that of the seven loci (0.993 and 0.999). After discarding the two loci that deviated from Hardy–Weinberg equilibrium in experimental verification, accurate probability of progeny assigned to their fathers and mothers with both parents unknown was 68% and 59% in CF (closely related families), and 76% and 68% in DF (distant related families), respectively, which were much lower than those predicted by the simulations. When the COI marker was used in combination with microsatellite markers, 32 out of 39 offspring that were not previously assigned to their correct parents were assigned to their correct mothers, and 29 doubtful offspring could be assigned to their correct fathers. Consequently, with the combination of the two kinds of markers, the accurate probability of progeny that can be correctly assigned to their true fathers and mothers was increased to 89% and 92% respectively. We suggest that the use of microsatellite markers and COI marker will greatly improve the efficiency of parentage determination in closely related families in the clam.

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1. Introduction

Owing to the dramatic decrease in marine resources caused by over-exploitation and pollution, domestic culture is becoming essential for the sustainable management of natural resources and increasing production of marine species. The clam, *Meretrix meretrix*, is an important aquaculture species in China. In the last decade, selective breeding programs were developed to provide cultivated seeds with better performance than the wild seeds (Liu et al., 2006). However, the detrimental inbreeding effects in such breeding programs could lead to degeneration of the valuable traits, such as reproductive capacity and physiological efficiency (Falconer and Mackay, 1996), whereas heterosis is displayed when the offspring performance exceeds the average performance of their parents, especially when the parents are more genetically different (Shikano and Taniguchi, 2002). Thereby, it is essential to minimize inbreeding depression, maximize heterosis, and gain reliable heritability of the target traits in the selective breeding programs of clam. However, the difficulty to maintain the pedigree information while eliminating environmental factors in mixed families is one of the most significant barriers in developing a breeding program. Routinely, families are raised in separate tanks until they are large enough for physical tagging (Jerry et al., 2001). It not only requires a lot of spaces and labors, but also introduces environmental effects which confound the analyses of genetic effects in different families (Bagley et al., 1994; Coman et al., 2002; Herbinger et al., 1999).

Along with the development of DNA molecular technologies, microsatellites as a kind of highly polymorphic co-dominant genetic markers has become an essential tool for parentage identification and genetic analyses in aquatic species (Blouin et al., 1996; Ferguson and Danzmann, 1998; Herbinger et al., 1995; O'Reilly et al., 1998; O'Reilly and Wright, 1995; Perez-Enriquez et al., 1999). Parentage determination using microsatellites has overcome the limitation of physical tags, allowing different families to be stocked together and assigned to their origin family without physical tagging (Garcia de Leon et al., 1998; Jerry et al., 2004; Norris et al., 2000).

However, some problems still exist, mainly regarding the optimal number of microsatellite loci required, the optimal level of polymorphism at each locus, and the treatment of genotyping errors in parentage analyses (Castro et al., 2004; Pompanon et al., 2005).

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Recently, a common trend is to screen the most efficient microsatellite markers while reducing the number of markers as much as possible (Bernatchez and Duchesne, 2000; Fessehaye et al., 2006; Porta et al., 2006), and some softwares are developed to simulate the performance of microsatellite markers in parentage assignment. However, the accuracy for assignment in experimental performance is much lower than that predicted by simulation (Dong et al., 2006; Jerry et al., 2004). As in other mollusks, due to technical limitations such as amplification failure (incomplete DNA purification and null alleles) and incorrect genotyping large deficits in heterozygotes were detected at many microsatellite loci (Hedgecock et al., 2004, 2007; Kijewski et al., 2009; Panova et al., 2008). Furthermore, with the increasingly complexness of the breeding systems such as in the closely related families, increases in the number of microsatellite markers are not enough to fulfill parentage identification correctly. Consequently, improved approaches should be developed for parentage identification. In the present study, we developed an effective method for parentage determination in the closely related families in the clam *M. meretrix*.

The gene sequences encoding the first subunit of mitochondrial cytochrome oxidase (COI) have abundant sequence variance within species. Owing to the benefits of COI for species identification (Hebert and Gregory, 2005), it has been shown to be suitable for the identification of a range of taxa, including gastropods (Remigio and Hebert, 2003), springtails (Hogg and Hebert, 2004), butterflies (Hebert et al., 2004a), birds (Hebert et al., 2004b), mayflies (Ball et al., 2005) and fish (Ward et al., 2005). In addition, mitochondrial genes have the pattern of maternal inheritance, so COI marker could be utilized to detect maternal parents in the parentage assignment. Furthermore, according to the computer simulation, when one parent is known before parentage analyses, the accuracy of assignment can be increased greatly. Therefore, the maternal parents' information derived from the abundant variance of COI can be very useful to improve the accuracy and overcome the limitation of microsatellite markers alone. Our investigation is the first attempt to combine the advantages of microsatellite markers with the characters of COI in parentage determination in closely related families.

2. Materials and methods

2.1. Family establishment

In early July, 2007, 25 families of the clam *M. meretrix* were produced according to a nested half-sib mating design (Wang et al., 2011). In brief, after the selected clam brood stocks were gradually conditioned in seawater from 20 to 26 °C for 10 days at the hatchery laboratory of the Zhejiang Mariculture Research Institute (Wenzhou, China), each mature female or male was induced to spawn in separate containers filled with seawater after being exposed to air for 4 h. After spawning, full- and half-sib families were produced according to a nested half-sib mating design. After half a year, nine full-sib families nested within three half-sib families were selected for parentage determination. The family tree of the nine families was shown in Fig. 1. The parent clams were frozen and stored at −20 °C separately.

2.2. Sampling of families and DNA extraction

Ten individuals were selected randomly from each of nine families, and a total of 90 individuals with individual identification (ID) were chosen as sample I for tracking parentage information. In order to compare the performance of microsatellite markers for parentage identification in closely related families (CF) and distant related families (DF), we constructed two groups using the nine families: the CF contained the families nested within half-sib families (J_9H_{36}, J_9H_{33}, X_7S_{27}, X_7H_{28}, J_5H_{17} and J_5H_8), and the DF contained full-sib families

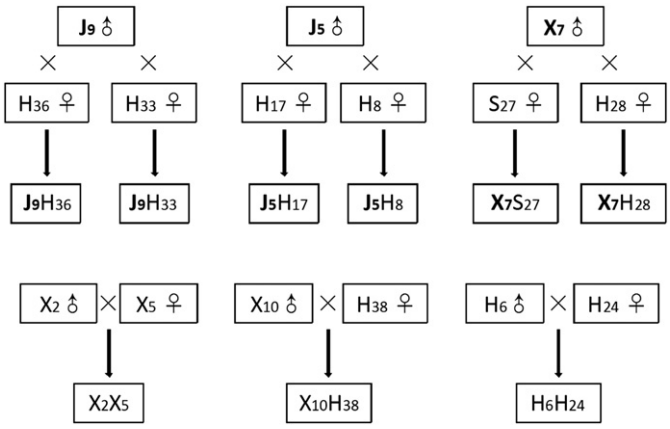

Fig. 1. A family tree of the nine families: the male J_5, J_9, and X_7 were used to construct half-sib families respectively.

(X_2X_5, H_6H_{24}, $X_{10}H_{38}$, X_7H_{28}, J_5H_{17} and J_9H_{36}). The 15 true parents (9 mothers and 6 fathers) with other ten female and ten male clams selected randomly from a cultured population as candidate parents (totally 35 individuals) were chosen as sample II. Parentage analysis was performed with CF (or DF) and sample II assuming no known parentage relationship between sample I and II.

Genomic DNAs of the candidate parents and their offspring were extracted from the muscle using a conventional hexadecyl–trimethyl–ammonium bromide (CTAB) protocol and resuspended in TE buffer (10 mM Tris–HCl pH 7.6, 0.1 mM EDTA) for parentage assignment.

2.3. Microsatellite amplification and evaluation

Seven polymorphic microsatellite loci (*MM*02, *MM*09, *MM*10, *MM*11, *MM*14, *MM*15, and *MM*16) (Table 1) developed in our laboratory were amplified in the sample II. All polymerase chain reactions (PCRs) consisted of a final volume of 20 μl containing 0.5 μM of each primer, 0.5 units of *Taq* DNA polymerase (Promega), 40 μM of each dNTP (Takara), 1.5 mM $MgCl_2$, 1× PCR buffer, and approximately 30 ng of DNA template. Thermal cycling was carried out in a MJ PCR-200 thermal cycler (Bio-Rad) under the following conditions: initial denaturation for 4 min at 94 °C, 32 cycles of denaturation at 94 °C for 50 s, annealing temperature (Table 1) for 40 s, and extension at 72 °C for 40 s, and a final extension step at 72 °C for 10 min. PCR products were electrophoresed on 8% non-denaturing acrylamide gels with pBR322 (MBI) as a standard DNA marker. Fragments were stained with ethidium bromide and visualized under UV light. Individual diploid genotypes were manually identified using Quantity One version 4.5 software (Bio-Rad).

Raw genotypes of sample II were tested for genotype errors in microsatellite data using Micro-Checker (Oosterhout et al., 2004), which can reveal the evidence of null alleles or scoring error due to allele-stuttering in our data set. Then they were analyzed for deviation from Hardy–Weinberg equilibrium at each locus based on Fisher's exact test using the Markov-chain method (Markov-chain length, 100,000; dememorization, 10,000) using Cervus Version 3.0 (Kalinowski et al., 2007), and for linkage between loci using Genepop Version 4.0.10 (Raymond and Rousset, 1995; available at http://genepop.curtin.edu.au). After these analyses, the loci that revealed deviation from Hardy–Weinberg equilibrium, linkage between loci, and evidence of null alleles were discarded from the parentage assignment.

The left loci were amplified in the sample I, and Micro-Checker was also used to test the genotypes of sample I to reveal evidence of null alleles or scoring error due to allele-stuttering.

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Table 1
Characteristics of the seven polymorphic microsatellite loci isolated from clam *M. meretrix*.

Locus	Primer sequences (5′-3′)		Size (bp)	Ta (°C)	GenBank accession no.
	Forward primers	Reverse primers			
*MM*02	GACAATAGCAGGACGATTATA	CGCGTGAAGTAAGTCCA	249–334	48 °C	GU250832
*MM*09	GGGTCTTGCAAGCGACATA	AAGAACAAGCAATAAGGCAT	240–330	53 °C	GU250838
*MM*10	GCGTAAAATGCGTATCAGA	CACCCTAGAAACCCCAGAG	227–324	51 °C	GU250845
*MM*11	GTGAGACATTCCTCTAACGG	ATGACTGACTTATTTCGGGG	242–340	54 °C	GU250846
*MM*14	TCAGGGATAACGGTGACAAT	GCAGTGAACCCACAACAGAA	226–315	58 °C	GU250841
*MM*15	GGTAAGAATGCCTGGAACA	AGGGATAACGGTGACAATG	152–291	57 °C	GU250842
*MM*16	TACCACCACCAACACCAT	AACTTCAGAAATCGTTATCC	169–250	50 °C	GU250843

Ta, annealing temperature.

2.4. Computed simulation analyses based on microsatellite markers

Simulation analysis was performed to estimate the probable performance of the microsatellite loci for parentage identification in *M. meretrix*. The simulations were based on the allele frequency information from the seven microsatellite markers of the sample II. Parental genotypes were generated assuming no deviation from Hardy–Weinberg equilibrium and no linkage between loci. Progeny genotypes were created based on parental genotypes assuming mutation or transmission error rate of 2% between parents and their progeny. In these cases, simulations were performed using the program Cervus 3.0 (Kalinowski et al., 2007). The combined probability of exclusion over loci with the genotypes of both parents unknown was termed as Excl 1 probability, and with known genotype of one parent was termed as Excl 2 probability.

2.5. Parentage analysis in mixed families by microsatellite markers

Parentage assignment for the mixed families (CF or DF) was estimated using the likelihood-based approach with the program Cervus 3.0. In this program, likelihood rations were calculated for parentage determination, and a statistic delta is defined as the difference in LOD scores between the most likely candidate parent and the second most likely candidate parent. Delta is used to assess the reliability of assigning parentage to the most likely candidate parent. The candidate parent with the highest (most positive) LOD score was considered as the true parent. The results were compared to the individual identification (ID), in order to calculate the correct probability.

2.6. Determination of maternal information by COI

In order to verify the feasibility of using mitochondrion COI marker combined with optimal number of microsatellite markers to increase the assignment accuracy in closely related families, we used the COI marker to determine the maternal parents of the offspring that were not assigned to their correct parents in the above parentage analysis. DNA templates of each individual from the candidate maternal parents and these offspring were amplified using specific primers LCO (5′-GGTCAA-CAAATCATAAAGATATTGG-3′) and HCO (5′-TAAACTTCAGGGTGAC-CAAAAAATCA-3′) (Folmer et al., 1994). All reactions were up to a final volume of 50 μl containing 1.25 μM of each primer, 1.25 units of *Taq* DNA polymerase (Promega), 100 μM of each dNTP (Takara), 3.75 mM $MgCl_2$, 1× PCR buffer, and approximately 100 ng of DNA template. Thermal cycling was carried out in a MJ PCR-200 thermal cycler (Bio-Rad) under the following conditions: initial denaturation for 4 min at 94 °C, 32 cycles of denaturation at 94 °C for 50 s, annealing at 57 °C for 40 s, and extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Reaction products were examined on a 2% agarose gel for 30 min, after which they were sequenced by Sangon Company (Shanghai, China). After the sequences were confirmed and edited manually, multiple alignments were performed using BioEdit Sequence Alignment Editor (Hall, 1999). After the maternal information was obtained, parentage assignment was performed in the candidate paternal parents and the doubtful offspring with the Cervus 3.0 program.

3. Results

3.1. Evaluation for the microsatellite loci

The genetic parameters calculated from the genotypes of sample II with Cervus indicated that the seven microsatellite loci have high polymorphism (Table 2). The mean observed heterozygosity (H_o), expected heterozygosity (H_e), and polymorphism information content (PIC) were 0.725, 0.839 and 0.799, respectively. No linkage was detected among the seven microsatellite loci. The locus *MM*02 and *MM*10 were found to be deviated from Hardy–Weinberg equilibrium (HWE) ($P<0.05$) through exact tests after sequential Bonferroni correction. Deficits of heterozygotes and null alleles were detected at these two loci, as their H_o was obviously lower than their H_e. After discarding these two loci, the mean H_o, H_e, and PIC of the five left loci were higher than those of the seven loci (Table 2).

3.2. Computed simulation based on microsatellite loci

Probability of exclusion per locus ranged from 0.356 to 0.597 when information of both parents was unavailable (Excl 1) and from 0.534 to 0.749 when one parent was known (Excl 2) (Table 3). The combined probabilities of exclusion were calculated with different subsets of polymorphic loci (from 1 to 7 loci) in the simulations. The relationship between the number of loci and combined probability of exclusion was showed in Fig. 2. The result of simulations indicated that the more markers were used, the higher accuracy would be gained for parentage assignment. Simulations demonstrated that combined exclusion probability of the five microsatellite markers was 0.991 and 0.995 for Excl 1 and Excl 2 respectively, which were not much lower than those of the seven loci (0.993 and 0.999).

Table 2
Summary statistics of seven microsatellite loci used in simulation study of *M. meretrix*.

Locus	*k*	H_o	H_e	PIC
*MM*02[a]	10	0.517	0.797	0.749
*MM*09	11	0.798	0.833	0.791
*MM*10[a]	11	0.424	0.776	0.719
*MM*11	17	0.817	0.828	0.789
*MM*14	19	0.853	0.891	0.863
*MM*15	16	0.829	0.868	0.837
*MM*16	13	0.836	0.877	0.847
Average				
7 loci	13.9	0.725	0.839	0.799
5 loci	15.2	0.827	0.859	0.825

k, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; PIC, polymorphic information content.
[a] Indicated the locus discarded from the parentage determination.

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Table 3
The exclusion probabilities of the microsatellite loci from the simulations.

Locus	Excl 1	Excl 2
*MM*02	0.397	0.578
*MM*09	0.461	0.636
*MM*10	0.356	0.534
*MM*11	0.459	0.635
*MM*14	0.597	0.749
*MM*15	0.548	0.709
*MM*16	0.566	0.725
CEP		
7 loci	0.993	0.999
5 loci	0.991	0.995

Excl 1, exclusion probability without known parents; Excl 2, exclusion probability with genotype of one parent known. CEP, combined exclusion probability. The five loci used for calculating combined exclusion probability did not contain the locus *MM*02 and *MM*10 that deviated from Hardy–Weinberg equilibrium.

3.3. Pedigree analysis in mixed families

The genotypes at five loci of CF and DF were used to construct Databank 1 and Databank 2 respectively, and the genotypes of males and females from sample II were used to construct two data files respectively (one for maternal parents and the other for paternal parents). For CF (or DF), the offspring databank and the two candidate parent data files were input to the Parentage Analysis process of Cervus 3.0, and the results were exported into a table of Excel. By comparing the assigned results with the individual identifications, the accurate probabilities of progeny assigned to their true fathers and mothers were 68% and 59% for CF, and 76% and 68% for DF, respectively, which were significantly lower than those predicted by the simulations. After the parentage analysis for mixed families using the five microsatellite markers, a total of 39 individuals in sample I were not assigned to their correct parents.

3.4. Parentage identification with known maternal information

The mitochondrion COI barcoding regions, a 658 bp fragment, of the candidate maternal parents and the previously undetermined individuals in sample I were sequenced. After multiple alignments of these sequences, a high level of variance was detected among maternal parents. Partial result of multiple alignments is shown in Fig. 3. Fig. 3 showed that sequences of the offspring (H_8-3, H_{33}-4, H_{36}-4, S_{27}-2, X_5-7 and X_5-9) were consistent with their mother H_8, H_{33}, H_{36}, S_{27}, and X_5, respectively. According to the multiple alignments, 32 of 39 doubtful offspring were assigned to their mothers. When the correct information of their maternal parents based on the COI marker and the five microsatellite markers were combined, 29 previously undetermined offspring were assigned to their correct paternal parents, using the Parentage Analysis process of Cervus 3.0. Consequently, with combination of the two kinds of markers, accurate probability of progeny assigned to their true fathers and mothers is increased to 89% and 92%, respectively.

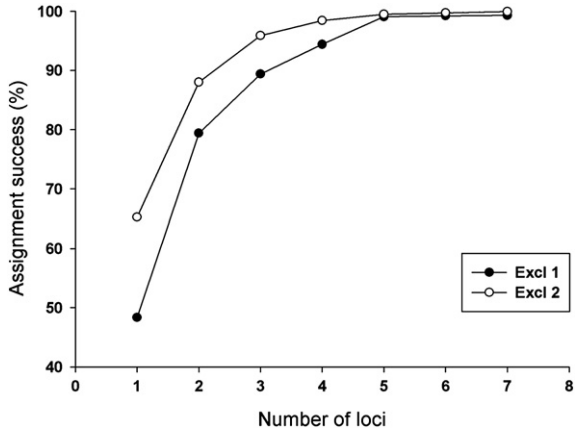

Fig. 2. Relationship between the number of microsatellite loci and assignment success of progeny to correct parents: combined exclusion probability with no known parent (Excl 1) and with one parent known (Excl 2).

4. Discussion

Estimating heritability is essential for identification of additive genetic variability in selective breeding programs, whereas to clarify genealogical relationships among individuals cultured in the same environment has become a challenge for breeders of aquatic animals such as the clam. Physical tags have been traditionally used in parentage determination in several aquaculture species (Arce et al., 2003; Caceci et al., 1999; Jerry et al., 2001; Linnane and Mercer, 1998; Uglem et al., 1996). However, the application of physical tags in the clam is limited, because tagging is a labor-intensive process and especially hard in early mix-rearing families. Therefore, reliable genetic markers are necessary to trace genealogies and estimate covariance between relatives (Falconer and Mackay, 1996). The utilization of microsatellite markers has demonstrated that the pedigree of mixed families could be determined by a few loci (Herbinger et al., 1995), based on Mendel's law to exclude doubtful parents. Since more polymorphic microsatellite markers are required for parentage determination in closely related families in the clam, a mollusk with high frequencies of null alleles, we feel a need to search for an effective method for tracing genealogies in this species. Wilson and Ferguson (2002) emphasized that simulation approaches are useful in evaluating the probable performance of a set of microsatellite loci. Simulations in many previous researches suggested microsatellite markers are obviously potential for application in parentage assignment (Ayres and Powley, 2004; Zou et al., 2010). In addition, the COI sequence has a high level of variance, and COI from mitochondria also has the character of maternal heredity. So we attempt to combine the two markers for parentage assignment in the clam *M. meretrix*.

In the present study, we evaluated the feasibility of parentage analyses based on both SSR and COI markets. We first evaluated the performance of seven microsatellite markers developed in our laboratory. Simulation results indicated that the parentage identification using the five loci was not much lower than that with the seven high polymorphism loci, after discarding the two loci with lower observed heterozygosity than their expected heterozygosity. Even with the five high polymorphic markers, the accurate probability of parentage assignment in mixed families of *M. meretrix* is still much lower than that predicted by the simulations. Discrepancies between the simulations and experimental results may be caused by many factors. Castro et al. (2004) suggested null allele, which is common in bivalve mollusks (Hedgecock et al., 2004), was a dominating source affecting the accuracy of parentage determination using microsatellite markers. This was verified in our research, as the assignment accuracy in closely related families was lower than that in distant related families, and null alleles were detected by the Micro-Checker program in offspring. Mutation was another effective factor but with lower possibility. Furthermore, scorning error due to allele-stuttering was an inevitable cause for mismatches. O'Reilly et al. (1998) found a scorning error rate of 2–3% per allele scored in their previous study. Consequently, when we select microsatellite markers to perform a large scale pedigree analysis, it is not only necessary to select high polymorphic markers, but also to consider the style of core sequence and the possibility of null alleles at these loci.

In consequence, when parentage in closely related mix-reared families was determined, more microsatellite markers with high polymorphism should be applied. This situation would lead to heavy

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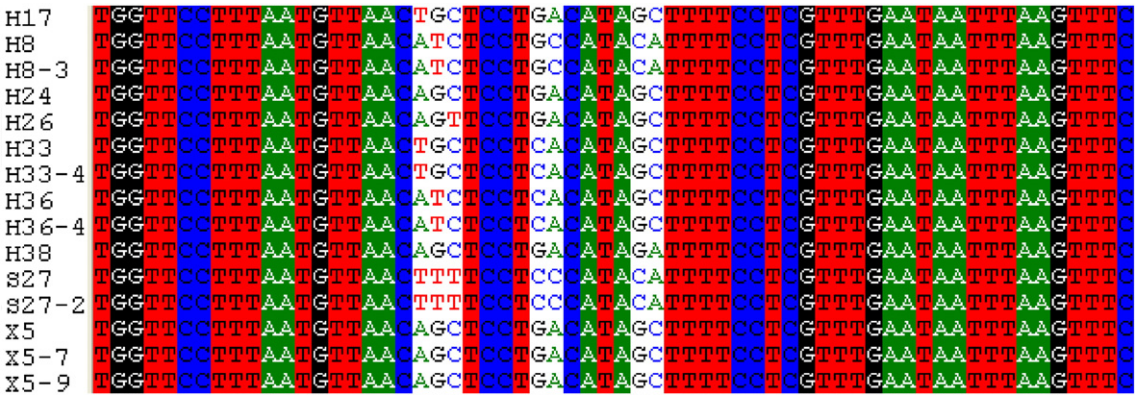

Fig. 3. Multiple alignments of COI sequences for candidate maternal parents and their progeny: H_8, H_{33}, H_{36}, S_{27}, and X_5 are the mothers of H_8-3, H_{33}-4, H_{36}-4, S_{27}-2, and X_5-7 (or X_5-9), respectively; others are the candidate maternal parents. The result of multiple alignments showed that there was high level of variance in sequence of COI in *M. meretrix*. The offspring and their mothers have the same sequence.

labor intensity, including isolation and characterization of more high polymorphic microsatellite markers, and amplification and analyses of genotypic data (Bernatchez and Duchesne, 2000; Sherman et al., 2004). Obviously the microsatellite markers alone could not meet our expectation for parentage determination in breeding programs of *M. meretrix*.

The COI marker was useful to help detect the maternal parent for the offspring. Here we validated that the utilization of COI marker can improve the accuracy of parentage determination in both full-sib families and half-sib families. Use of COI marker could overcome the limitation of microsatellite loci in mollusks to achieve higher accurate probabilities under real experimental conditions. Accordingly, in the present breeding programs of *M. meretrix*, the application of COI and microsatellite markers was proven to be an effective method for parentage assignment. It is worth to be mentioned that, along with the progress of breeding programs, the breeding population would normally be close in genetics, leading to the ineffectiveness of COI marker system with each generation as the number of founding females contributing their mitochondrial system to the next generation will be reduced. Consequently, application of this method should be cautious when the parentage identification was performed in half-sib families that were constructed from the same female and different males with high frequencies of null alleles at microsatellite loci, or the maternal parents were derived from the same mother and mated with a same male. However, before new methods are developed for parentage identification in the future, this method is still the most preferred methodology available now.

5. Conclusion

In the present study, we show that microsatellite markers combining with COI is an effective approach to determine the pedigree information in mix-reared families of clam *M. meretrix*. This method can greatly enhance the accuracy for parentage determination in half-sib families and reduce the number of microsatellite markers needed in breeding programs. Consequently, this effective method makes it possible to cultivate different families together for genetic improvement, because it not only can minimize environmental interference but also has high accuracy for parentage assignment.

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