Aquaculture 318 (2011) 223–228

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

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

Xia Lu [a,b], Hongxia Wang [a], Baozhong Liu [a,*], Jianhai Xiang [a]

[a] Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[b] Graduate School of the Chinese Academy of Sciences, Beijing 100039, China

ARTICLE INFO

Article history:
Received 15 July 2010
Received in revised form 5 May 2011
Accepted 5 May 2011
Available online 13 May 2011

Keywords:
Microsatellite marker
Mitochondrial cytochrome oxidase (COI)
Meretrix meretrix
Parentage determination

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).

* Corresponding author. Tel.: +86 532 82898696; fax: +86 532 82898578.
E-mail address: bzliu@qdio.ac.cn (B. Liu).

0044-8486/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2011.05.017

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

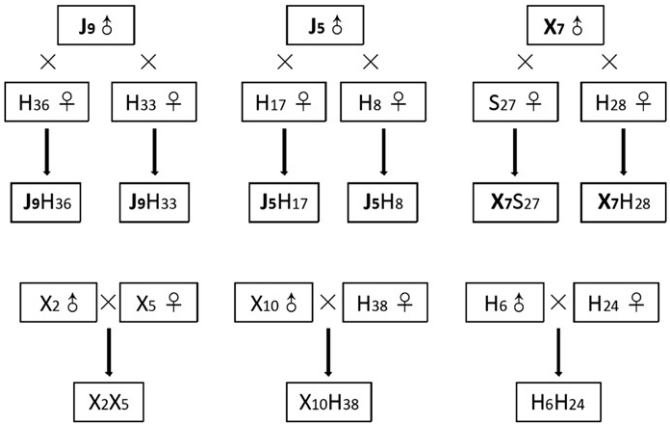

Fig. 1. A family tree of the nine families: the male J_5, J_9, and X_7 were used to construct half-sib families respectively.

(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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